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الجامعة الأردنيـــة
كلية الدراسات العليا

التاريـــخ: / /

نموذج رقم (١٦)
اقرار والتزام بالمعايير الأخلاقية والأمانة العلمية وقوانين الجامعة الأردنية وأنظمتها وتعليماتها لطلبة الدكتوراه

أنا الطالب: منال أحمد عبدالله عكة الرقم الجامعي: (٩٠٧٠٠٣٤)
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عنوان الاطروحة: ...
Evaluation of the efficacy of some immunomodulators and anti-cancer agents as potential therapeutic agents for endometriosis

اعلن بأنني قد التزمت بقوانين الجامعة الأردنية وأنظمتها وتعليماتها وقراراتها السارية المفعول المتعلقة باعداد اطروحات الدكتوراه عندما قمت شخصيا" باعداد اطروحتي وذلك بما ينسجم مع الأمانة العلمية وكافة المعايير الأخلاقية المتعارف عليها في كتابة الأطروحات العلمية. كما أنني أعلن بأن اطروحتي هذه غير منقولة أو مستلة من أطاريح أو كتب أو أبحاث أو أي منشورات علمية تم نشرها أو تخزينها في أي وسيلة اعلامية، وتأسيسا" على ما تقدم فانني أتحمل المسؤولية بأنواعها كافة فيما لو تبين غير ذلك بما فيه حق مجلس العمداء في الجامعة الأردنية بالغاء قرار منحي الدرجة العلمية التي حصلت عليها وسحب شهادة التخرج مني بعد صدورها دون أن يكون لي أي حق في التظلم أو الاعتراض أو الطعن بأي صورة كانت في القرار الصادر عن مجلس العمداء بهذا الصدد.

توقيع الطالب: منال عكة .... التاريخ: ١٣ / ٨ / ٢٠١١

EVALUATION OF THE EFFICACY OF SOME IMMUNOMODULATORS AND ANTI-CANCER AGENTS AS POTENTIAL THERAPEUTIC AGENTS FOR ENDOMETRIOSIS

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This Thesis was Submitted in Partial Fulfillment of the Requirements for the Doctor of Philosophy Degree in Biological Sciences

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COMMITTEE DECISION

This Thesis/Dissertation (Evaluation Of The Efficacy Of Some Immunomodulators And Anti-Cancer Agents As Potential Therapeutic Agents For Endometriosis) was Successfully Defended and Approved on August, 2011.

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و ما أوتيتم من العلم الا قليلا

سورة الإسراء :الآية ٨٥

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Manal Abbas

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A	Adenosine
AFS	American Fertility Society
AI	Aromatase inhibitor
AIF	Apoptosis-inducing factor
ATP	Adenosine triphosphate
AMP	3', 5' adenosine monophosphate
ASRM	The American Society for Reproductive Medicine
BAX	Pre-apoptotic factors
BCG	Bacillus Calmette-Guerin
Bcl-2	Anti-apoptotic factors
cAMP	Cyclic adenosine monophosphate
CAM	Chorioallantoic membrane
CB	Cannabinoid receptor
CD	Cluster of differentiation
cGMP	Cyclic 3',5' guanosine monophosphate
COCs	Combined oral contraceptives
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
CSF	Colony-stimulating factor
D_2	Ergocalciferol
D_3	Cholecalciferol
DAB	Diaminobenzidine
DMSO	Dimethylsulfoxide
D.W	Distilled water
DPBS	Dulbecco’s phosphate buffered saline
DPX	Distrene-Plasticizer-Xylene
E2	17 β-Estradiol
EPCs	Endothelial progenitor cells
ER	Estrogen receptor
ER-β	Estrogen receptor β
ERK	Extracellular regulated kinase
FDA	Food and Drug Administrattion
FLT3	FMS-like tyrosine kinase-3 receptor
FSH	Follicle stimulating hormone
GM-CSF	Granulocyte-macrophage colony stimulating factor
GMP	3',5'Guanosine monophosphate
GnRH	Gonadotropin releasing hormone
HDAC	Histone deacetylase
H& E	Haematoxylin and eosin
HeLa	Human epithelioid cervix carcinoma
HRP	Horseradish peroxidase
HSD17B1	17β-hydroxysteroid dehydrogenase 1
HSD3B2	3β-hydroxysteroid dehydrogenase 2

HMG-CoA	3-hydroxy-3-methylglutaryl–coenzyme A
IC_{50}	Median Inhibition Concentration
ICAM-1	Intercellular adhesion molecule 1
IgE	Immunoglobulin E
IFN-γ	Interferon- γ
IL	Interleukin
iNOS	Inducible nitric oxide synthase
i.p	Intraperitoneal
I-κB	Inhibitory protein κB
IKK	IκB kinase
JNK	c-Jun NH2-terminal Kinase
KIT	Stem cell factor receptor
LH	Luteinizing hormone
LNG IUS	Levonorgestrel intrauterine system
LPS	Lipopolysaccharide
LT	Leukotriene
LX	Lipoxins
MAPK	Mitogen-activated protein kinase
MAPKK	MAPK kinase
MMP	Matrix metalloproteinase
mTOR	Mammalian target of rapamycin
NaCl	Sodium chloride
NDGA	Nordihydroguaiaretic acid
NF-κB	Nuclear factor-κB
NK	Natural killer cells
NOAEL	No observable adverse effects levels
(NOD)-SCID	Non-obese diabetic mice
NSAID	Non steroidal anti inflammatory drugs
$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D
OVX	Oophorectomy
PBS	Phosphate buffer saline
PDE	Phosphodiesterase
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PFM	Positive-feed back model
PG	Prostaglandin
PI3K	Phosphatidylinositol 3-kinase
PK	Protein kinase
PKC	Protein kinase C
PTEN	Phosphatase and tensin homolog
RET	Rearranged during transfection
RIA	Radioimmunoassay
Hpf	High power field
r-hTBP1	Tumour necrosis factor-alpha-binding protein
RTK	Receptor protein-tyrosine kinase

SCC	Side-chain cleavage enzyme
SCID	Severe combined immunodeficient mice
SE	Standard error
SERM	Selective estrogen receptor modulators
SF1	Steroidogenic factor 1
sFlt-1	Soluble form of truncated VEGF receptor
SPRM	Selective progesterone receptor modulators
STAR	Steroidogenic acute regulatory protein
STAT3	Signal Transducer and Activator of Transcription 3
TGF-β	Transforming growth factor-β
Th1	T-helper-2
TNF	Tumor necrosis factor
TSG	Tumour suppressor gene
TUNEL	Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay
UV	Ultraviolet
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

EVALUATION OF THE EFFICACY OF SOME IMMUNOMODULATORS AND ANTI-CANCER AGENTS AS POTENTIAL THERAPEUTIC AGENTS FOR ENDOMETRIOSIS.

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ABSTRACT

Endometriosis is one of the most frequent diseases in gynecology. Currently, available medical therapies are unsatisfactory because of their side effects and because they compromise fertility. The aim of the present study was to discover new therapeutic agents for endometriosis based on current understanding of its pathogenic mechanisms, the advancement in the understanding of the role of the immune system and the similarity between endometriosis and cancer. The effect of nine compounds on regression of endometriotic implants was studied in a rat surgical model. β-caryophyllene (10 mg/kg, 30 mg/kg), nordihydroguaiaretic acid (masoprocol) (10 mg/kg), primaquine (10 mg/kg), sunitinib (sutent) (10 mg/kg), theophyllene (10 mg/kg, 15 mg/kg) and vitamin D (42 µg/kg) reduced cyst cross sectional area by 52.5%, 41.0%, 45.4%, 43.1%, 80.3%, 52.0%, 65.5%, 51.1% respectively. On the other hand, caffeine, citronellol and thymoquinone produced no statistically significant effect. Histologically, extensive fibrosis was detected in sunitinib-treated group with positive reaction in terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay indicating that apoptosis is a mechanism of action. The other effective compounds produced variable degrees of fibrosis and apoptosis in epithelial and stroma cells. Ultrastructural studies revealed the presence of fibrosis and degenerative changes in the stroma of the effective compounds. In aortic ring assay, caryophyllene exhibited anti-angiogenic activity. Interestingly, β-caryophyllene had no statistically significant effect on ovulation, implantation, mid-gestation, late-gestation and normal delivery on any of the studied parameters including implantation sites, adsorption sites, number of corpora lutea, number and weight of fetuses and newborns and estrogen and progesterone hormone serum levels in rats. Therefore, the active compounds may represent promising alternatives to the current medical therapies which interfere with fertility.

Introduction

1.1. Definition of Endometriosis

Endometriosis is a benign gynecological condition defined by the ectopic presence of both endometrial glands and stroma (D'Hooghe *et al.*, 2009). The usual location of endometriosis is in the pelvis mostly in the ovaries and the dependent areas covered with peritoneum such as the pouch of Douglas (a deep peritoneal recess between the uterus and the upper vaginal wall anteriorly and the rectum posteriorly called also cul-de-sac) and the uterosacral ligaments (Figure 1) (Murphy, 2002). Endometriotic lesions were also described in extrapelvic sites, including anterior abdominal wall, surgical scars, diaphragm, omentum, small intestine, appendix, lung, urinary tract, musculoskeletal, and neural systems (Kim and Adamson, 2008).

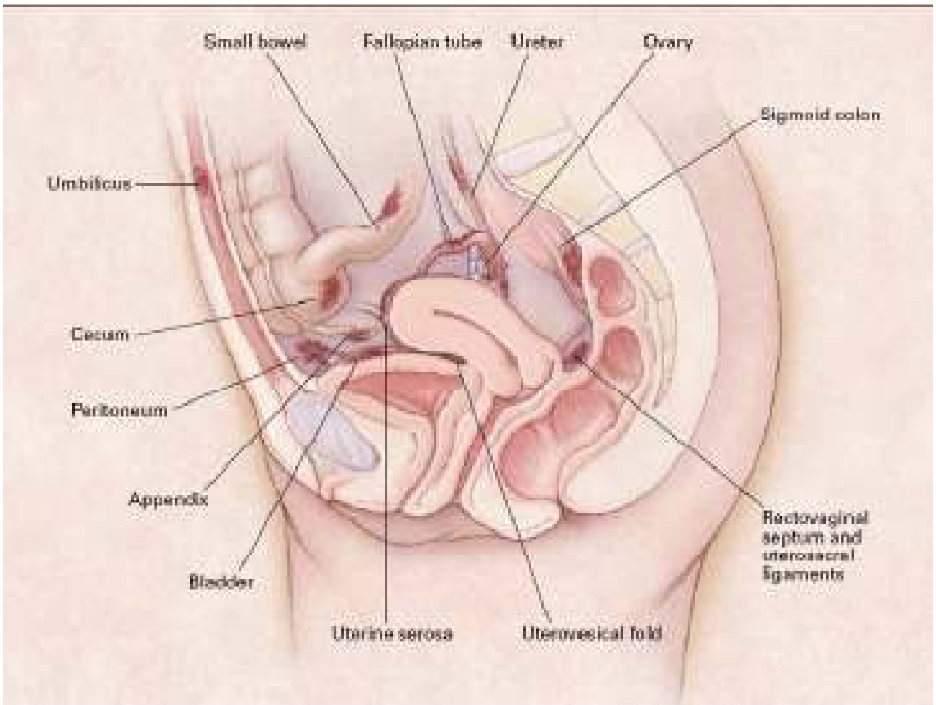


Figure 1. Common locations of endometriotic lesions within the peritoneal cavity. Dark red patches in the figure indicate the presence of an endometriotic lesion for example on the ovary, bladder, cecum, bladder, uterine serosa, rectovaginal septum, .. etc. (Olive and Pritts, 2001).

Endometriosis is classified into three different types: ovarian, peritoneal and rectovaginal (or deep-infiltrating) endometriosis (Nisolle and Donnez, 1997). Ovarian endometriosis is demonstrated as large hemorrhagic cysts of the ovary (Figure 2 A, B). On the other hand, peritoneal endometriosis usually appears as superficial implants that are red, black or white in color (as seen during laparoscopy). The color of the lesion depends on the level of angiogenic activity, hemorrhage and fibrosis (Murphy, 2002). Red lesions are highly vascularized and are assumed to bleed. White lesions include fibrotic material and are supposed to be a scar tissue while black lesions often contain clotted blood. Lesion size ranges from pinhead up to large, egg-shaped cystic formations (ASRM, 1997). The third type, rectovaginal or deep invasive endometriosis, is usually found in the pouch of Douglas behind the uterus, in the rectovaginal septum or on the bladder. Deep-infiltrating endometriosis is sometimes an aggressive disease associated with massive fibromuscular reactions, often leading to ureteral obstruction and loss of kidney function (Donnez *et al*., 2002).

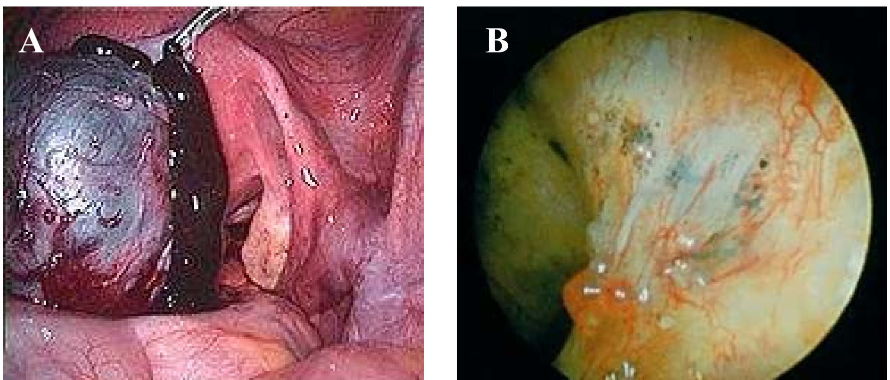


Figure 2. Laparoscopic view of endometriotic implants
(A) Ruptured Chocolate Cyst in Ovary From: http://en.wikipedia.org/wiki/Endometriosis_of_ovary
(B) An endometriotic implant in the cul-de-sac that exhibits multiple vesicles, "powder burn" lesions, and mild fibrosis (Kim and Adamson, 2008).

In 1997, an internationally accepted classification of endometriosis was published by The American Society for Reproductive Medicine (ASRM) in a revised version. This classification was based on a weighted point system, in which the following parameters were investigated: superficial or deep endometriosis, severity of adhesions (filmy or dense), obliteration of the pouch of Douglas and enclosure of the ovaries. According to this classification, four different stages of endometriosis should be distinguished: Stage I (Minimal): findings restricted to only superficial lesions and possibly a few filmy adhesions. Stage II (Mild): some deep lesions are present in the cul-de-sac. Stage III (Moderate): as stage II, plus presence of endometriomas on the ovary and more adhesions. Stage IV (Severe): as stage III, plus large endometriomas and extensive adhesions (ASRM, 1997). The detection of the phenomenon known as “kissing ovaries” in which ovaries are very closely glued together is a special indicator of severe endometriosis (stage IV) (Ghezzi *et al.*, 2005).

1.2. Signs and Symptoms of Endometriosis

I-Pain

Pains including dysmenorrhea (painful menstrual periods), dyspareunia (pain on intercourse) and dyschezia (pain during defecation) are associated with endometriosis (Story and Kennedy, 2004). Pain varies in severity from light to severe. It may progress and begin before to the onset of menses or become chronic and be noted throughout most of the menstrual cycle (Murphy, 2002). Dyspareunia is usually associated with rectovaginal and uterosacral ligament disease. Also, it may be associated with fixation of the uterus or ovary

by adhesions, especially if an endometrioma is found. On the other hand, bowel implants can cause rectal bleeding or obstruction (Kim and Adamson, 2008).

According to Sinaii *et al.* (2008), endometriosis is diagnosed in 40-70 % of cases in women with chronic pelvic pain. Interestingly, women with extensive endometriosis may have little or no pain symptoms. They may come to the attention of physicians because of associated infertility or as an incidental finding. Conversely, women with only minimal or mild involvement may manifest with disabling pelvic pain (Murphy, 2002).

II- Infertility

Infertility is a symptom strongly associated with endometriosis (D'Hooghe *et al.*, 2003; Matalliotakis *et al.*, 2008). Recent data from the Practice Committee of ASRM demonstrate that actually 30-50 % of all infertile women suffer from endometriosis (ASRM, 2004).

Infertility may be explained by mechanical interference with oocyte pickup and transport and altered tubal peristalsis if adhesions or significant anatomic distortion is present (Kim and Adamson, 2008). However, if anatomic distortion of the pelvis is abscent, the mechanism of infertility is poorly understood. Theories that try to explain the reason of infertility include altered folliculogenesis, ovulatory dysfunction, sperm phagocytosis, impaired fertilization, defective implantation, inhibition of early embryo development, luteal phase defects, and immunologic alterations (Grant, 1966; Kim and Adamson, 2008; Mahadevan *et al.*, 1983; Muscato *et al.*, 1982). In fact, the peritoneal cavity of women with endometriosis is associated with chronic inflammatory response. Peritoneal fluid white

blood cells are thought to interfere with fertility by exerting direct cytotoxic effects or by releasing cytokines and proteolytic enzymes into the pelvic milieu that can affect gamete function or embryo growth (Kim and Adamson, 2008).

III- Other Symptoms

Abnormal uterine bleeding has been frequently associated with endometriosis (Scott and Te Linde, 1950). However, most cases of bleeding were attributed to coexistent pathology rather than true dysfunctional uterine bleeding. For example, anovulation was reported in 17% of women with endometriosis (Soules *et al.*, 1976).

Uncommon symptoms are usually attributable to endometriosis involvement of atypical locations. Endometriosis located in the bladder may cause suprapubic pain, frequency, urgency, dysuria, and hematuria. Ureteral involvement may cause upper urinary tract symptoms such as flank pain or backaches (Kim and Adamson, 2008). Pulmonary involvement can result in pleuritic pain, pleural effusion, cough, hemoptysis, or pneumothorax (Foster *et al.*, 1981). Cyclic headaches or seizures may indicate brain lesions (Thibodeau *et al.*, 1987). Sciatica has been reported from endometriosis in the retroperitoneal space (Denton and Sherrill, 1955). Some symptoms such as pain or bowel obstruction may persist despite castration or menopause, usually from scarring or adhesions (Murphy, 2002).

1.3. Prevalence and Epidemiology of Endometriosis

Endometriosis was introduced clinically at the turn of the 19th century, as ovarian ‘‘chocolate cysts’’, and as adenomyosis externa. In 1899, smaller lesions of endometriosis

were described. After the introduction of endoscopy in the late 1960s, black-puckered endometriosis lesions were recognized to be a frequent observation in women with pain and/or infertility. In the 1980s, non-pigmented endometriotic lesions were described. As a result, the reported prevalence of the disease increased even further. Later on in the 1990s, the awareness grew that deep infiltrating endometriosis was more frequent than thought before (Koninckx, 1998).

Endometriosis is one of the most frequent diseases in gynaecology, affecting 10-15 % of all women in the reproductive age and as many as 40 to 50% of all women with infertility (Nothnick and Zhang, 2009). Although no consistent information is available on the incidence of the disease, temporal trends suggest an increase among women of reproductive age. This could be explained—at least in part—by changing reproductive habits. Several epidemiological studies have indicated that nulliparous women and women reporting short and heavy menstrual cycles are at increased risk of developing endometriosis (Vigano *et al*., 2004).

It is worth noting that there are no published studies on representative samples of the general population. So, it is difficult to compare estimates of prevalence because the published studies include women with different conditions, and are conducted in centers that apply different diagnostic criteria and exhibit different levels of clinical interest in endometriosis (Vigano *et al*., 2004).

1.4. Risk Factors

I- Age: The progression of endometriosis shows a strong relationship to estrogen levels; the disease incidence rises sharply at menarche then decreases in menopausal women (Uchiide *et al*., 2002).

II- Social class and race: Neme *et al*. (2002) reported a greater frequency of endometriosis among women of higher social class. However, this might be the result of a diagnostic 'bias', i.e. greater attention is paid to pelvic pain or infertility in women of higher social class. The same diagnostic bias might explain the higher frequency of the disease among white women (Vigano *et al*., 2004).

III- Menstrual and reproductive factors: In three case-controlled studies, greater exposure to menstruation because of longer flows, shorter cycles, or lower parity appears to increase the risk of endometriosis (Candiani *et al*., 1991; Cramer *et al*., 1986; Darrow *et al*., 1993). Parity is inversely associated with the risk of endometriosis (Candiani *et al*., 1991). However, the few available epidemiological data have not generally shown any relationship between age at first pregnancy, spontaneous abortion, and endometriosis (Cramer *et al*., 1986).

IV- Oral contraceptive use: According to Sangi-Haghpeykar and Poindexter (1995), the risk of endometriosis was lower among current oral contraceptive users. However, data regarding the association between oral contraceptive use and endometriosis are conflicting. It has been suggested that although oral contraceptive use temporarily suppresses

endometriosis, previous oral contraceptive use could increase the risk of the disease (Vigano *et al*., 2004). A reasonable explanation is that the treatment with oral contraceptive does not cure endometriosis and that ectopic endometrial implants survive, although in atrophic form, ready for reactivation when treatment stops (Nisolle-Pochet *et al.,* 1988).

V- Family history and genetic factors: The risk of endometriosis was increased sixfold to sevenfold for relatives of an affected person compared with controls (Kim and Adamson, 2008). Furthermore, endometriosis might be more severe in women with a family history (Malinak *et al*., 1980).

VI- Smoking, diet, and other lifestyle issues: Factors such as smoking that reduce endogenous estrogen levels may have an inverse association with the risk of endometriosis (Candiani *et al*., 1991). The relationship between an increased risk of endometriosis and alcohol, coffee, smoking, or a diet rich in saturated fats is controversial (Vigano *et al*., 2004). A moderate intake of alcohol is related to increased levels of estrogens. A high intake of saturated fats is associated with increased risk of other benign or malignant gynaecological conditions (La Vecchia *et al*., 1987). On the other hand, regular physical activity might be linked with lower levels of estrogens and reduced endometriosis risk (Vigano *et al*., 2004).

VII- Body mass index: Overweight women are at lower risk of endometriosis (Cramer *et al*., 1986). A possible explanation for this is that women with an increased body mass index have more irregular menstrual cycles and increased rates of anovulatory infertility (Vigano *et al*., 2004).

VIII- Environmental Pollutants: Major concern has been raised with regards to a possible relationship between exposure to environmental contaminants and the development of endometriosis (Kitajima *et al*., 2004). Environmental toxicants could influence the growth and development of endometriosis through hormonal mimicry or altered immune responses (Cummings and Metcalf, 1995).

Dioxins, chlorinated hydrocarbons, are suspected to contribute to the genesis of endometriosis. They are by-products of industrial processes (e.g. waste incineration or iron and steel industries) (Heilier *et al*., 2008). Although environmental concentrations of dioxins are generally low, these chemicals are resistant to degradation and, due to their lipophilic nature, bioaccumulate and biomagnify at higher levels within the food chain. Approximately 80% of human environmental exposure to dioxins comes from the consumption of fat-containing foods such as milk, meat, and fish (Grassman *et al*., 1998).

Among the numerous dioxin-like environmental contaminants, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is often considered to be the prototypical disruptor of steroid action within endocrine sensitive tissues, affecting steroid receptor levels as well as steroid metabolism and serum transport. In addition, it dramatically affects the endocrine system. TCDD exposure also affects the expression of multiple cellular signaling systems that regulate key elements of the immune system **(**Nayyar *et al*., 2007**)**. It has been demonstrated that the incidence and severity of spontaneous endometriosis in rhesus monkeys was increased following dietary exposure to TCDD (Cummings *et al*., 1996). However, a short-term exposure to TCDD failed to increase the growth of endometriotic lesion in mice.

Similarly, the potential for estrogenic contaminants to facilitate the progression of endometriosis has been demonstrated in mice with surgically induced endometriosis treated with methoxychlor, a contaminant with estrogenic activities (Cummings and Metcalf, 1995). Also, 4-chlorodiphenyl ether, at doses relevant to human exposure through fish diet, stimulated the growth of surgically induced endometriosis in mice. This compound has structural similarity to a well-known man-made estrogen, diethylstilbestrol which suggests that 4-chlorodiphenyl ether mimics estrogen effects on endometriotic tissues because the endometrial tissues are dependent on estrogenic support for survival (Yang *et al*., 1997).

1.5. Pathogenesis of Endometriosis

The main etiopathogenetic theories are the transplantation or implantation (Sampson, 1927), coelomic metaplasia (Meyer, 1919) and induction theories (Merrill, 1966). Sampson's theory (also known as the theory of retrograde menstruation and implantation) proposes that viable endometrial tissue is refluxed through the fallopian tubes during menstruation and implants on peritoneal surface or pelvic organs (Sampson, 1927). This theory is the most widely accepted aetiology of endometriosis today (Laschke *et al*., 2006).

Substantial evidence exists to support the implantation hypothesis. Studies have confirmed the occurrence of retrograde menstruation and have shown it to be a very common phenomenon among women (76–90% of women) (Liu and Hitchcock, 1986).

Early evidence of viability of endometrial cells in the menstrual flow was demonstrated by supravital staining (Geist, 1933) and by *in vitro* growth of menstrual endometrium

(Keettel and Stein, 1951). In an experiment to determine the viability of discharged endometrial tissue, menstrual effluent from the second day of menstruation was injected into subcutaneous abdominal fat. After 90–180 days, the injection site was excised for histologic examination and found to have endometriosis (Ridley and Edwards, 1958). In another experiment using a monkey model, extensive endometriosis developed in the peritoneal cavity in one half of the monkeys after diversion of menstruation into the peritoneal cavity (TeLinde and Scott, 1950).

Endometrium was successfully implanted experimentally and grown within the peritoneal cavity. This has been demonstrated in animals by the viability of surgically transplanted endometrium (Schenken and Asch, 1980). Similarly, D'Hooghe *et al*. (1995) injected menstrual endometrium in a retroperitoneal location in four baboons. All developed endometriosis, and three of the four had progression of the implants after 12 months of observation (D'Hooghe *et al*., 1995)

Human studies also gave more evidence for Sampson's theory. In women with obstruction to the normal outflow of menstrual fluid, a higher incidence of endometriosis was observed (Olive and Henderson, 1987). Also, other observations providing circumstantial evidence in support of retrograde menstruation include the anatomic distribution of endometriosis. The most frequent sites of implantation are the dependent areas of the pelvis (*i.e.* ovary, posterior and anterior cul-de-sac, and uterosacral ligaments), which are most likely to be affected by menstrual reflux from the fallopian tubes (Jenkins *et al*., 1986).

Sampson's theory fails to explain the presence of endometriosis in such remote areas outside the peritoneal cavity, as the lungs, skin, lymph nodes and breasts (Bulun, 2009). Dissemination of endometrial cells through the lymphatic and vascular system has been demonstrated and has been used to explain the presence of endometriosis at these various locations (Javert, 1949). Given the usual anatomic distribution of endometriosis, however, these routes of endometrial cell dissemination are not thought to be common (Kim and Adamson, 2008).

Though retrograde menstruation is a quite common phenomenon among women, endometriosis is found in only about 10% of women in reproductive age. So other factors, such as genetic predisposition as well as immunological alterations may determine women’s susceptibility to endometriosis (D’Antonio *et al*. 2000, Dmowski *et al*., 1981). Alterations in the immune response may prevent adequate disposal of menstrual debris, possibly increasing the chances of endometriosis development. Alternatively, excessive menstrual reflux may overwhelm the capacity of an intact immune system to remove endometrial tissue or suppress its growth (Kim and Adamson, 2008).

The coelomic metaplasia theory proposes that formation of endometriomas in the ovary or recto-vaginal endometriosis is caused by metaplasia of the coelomic epithelium (Meyer, 1919). This theory was based on embryological studies that illustrate that peritoneal mesothelium, the müllerian ducts, and the germinal epithelium of the ovary are all derived from celomic wall epithelium (Gruenwald, 1942). So, it was suggested that these tissues retain the capacity for further differentiation or that the peritoneal mesothelium and germinal epithelium contain undifferentiated cells capable of developing into functioning

endometrium. Celomic epithelium can be found in the thoracic cavity, umbilicus, and limb buds, which can explain the occurrence of endometriosis in these unusual ectopic sites (Kim and Adamson, 2008).

Clinical observations have cast further doubt on the validity of coelomic metaplasia theory. Endometriosis would be expected to occur in males as well as females if peritoneal epithelium could undergo metaplasia. The occurrence of endometriosis in males, however, has been reported in only a few cases of men receiving high-dose estrogen therapy for prostatic carcinoma (Pinkert *et al*., 1979). Despite the contribution of celomic tissue to the development of peritoneum and pleura, endometriosis is located primarily in the pelvis and not uniformly distributed in other sites where the celomic membrane is present. In light of these observations and the lack of experimental evidence supporting this theory, it is unlikely to be an explanation for the pathogenesis of endometriosis in most women (Kim and Adamson, 2008).

The induction theory of endometriosis is a combination of the transplantation and celomic metaplasia theories. This theory proposes that specific agents released from shed endometrium induce undifferentiated mesenchyme to develop into endometriosis. Experimental support for this theory exists from animal studies. In a study using rabbits, millipore filters containing endometrium, myometrium, or fat were implanted into animals (Merrill, 1966). The tissue surrounding the diffusion chambers containing endometrium were excised and found to have cysts lined with cells resembling

endometrial epithelium and endometrium-like glands. This finding was not observed in the tissue surrounding the myometrium or fat chambers.

In addition to these theories, direct extension of endometrium through the uterine wall, and uterotubal extension has been suggested (Kim and Adamson, 2008). The direct extension theory proposes that endometriosis arises from the invasion of endometrium through the uterine musculature. This theory was based on the observation that glandular structures of adenomyosis were in direct contact with eutopic endometrium. However, no conclusive evidence of the progression of endometrial invasion through the myometrium into the pelvis to present as endometriotic implants has been reported (Kim and Adamson, 2008).

The theory of uterotubal extension is a combination of the direct extension and transplantation theories, which suggests that endometriosis is a consequence of direct extension through the tubal wall (Kim and Adamson, 2008). A few studies have reported the presence of tubal endometriosis but did not determine whether ectopic endometrial implants were contiguous (Donnez *et al*., 1984).

1.6. Current Understanding of the Pathogenesis of Endometriosis

1.6.1 The Role of Immunological Alterations in the Pathophysiology of Endometriosis

Immunological alterations in endometriosis patients were regarded as secondary side effects for decades. Therefore, they have not been further evaluated as potential cause of the disease or possible target for therapeutical approaches (Eltern, 2008). However, in

recent years, the role of the immune system in the pathophysiology of endometriosis has increasingly gained attention (Osuga *et al*., 2011).

Immune status is suggested to play an important role in the initiation and the progression of endometriosis. In particular, immune cells in lymphoid lineage that comprised T and B lymphocytes and natural killer cells. Impairment of the cell-mediated immune response is thought to have a permissive effect on the implantation of refluxed endometrial cells, thereby increasing the chance of endometriosis development. Assuming natural killer (NK) cells target endometrial cells, abnormal NK cell function may result in inadequate clearance of refluxed endometrium from the peritoneal cavity (Hill, 1992). The cytotoxicity of circulating and peritoneal NK cell is reduced in women with endometriosis (Oosterlynck *et al*., 1991, Wilson *et al*., 1994). Furthermore, the ectopic endometrium secretes substances inhibiting NK cells (Hirata *et al*., 1994).

Numerous studies have shown reduced cytotoxic activity of T cells (Harada *et al*., 2001) and secretion of cytokines by helper T cells. These alterations were suggested to promote the disease (Osuga *et al*., 2011). In addition, abnormalities in B-cell function and a high incidence of abnormal autoantibodies in women with endometriosis have been reported in several studies. Antibodies to endometrial tissue may facilitate the growth of endometriosis by blocking the recognition sites required for T-cell-mediated destruction (Evers *et al*., 1993; Kim and Adamson, 2008).

Peritoneal macrophages increase in number and activation status in patients of endometriosis and, consequently, there is a higher release of macrophage-derived products, such as growth factors and cytokines, which could be involved in the pathophysiology of the disease (Halme *et al*., 1987, Szymanowski *et al*., 2009). Macrophages are thought to have a major role in the local immune process. In addition to removing red blood cells, tissue debris, and possibly endometrial cells, macrophages may contribute to the development of endometriosis by secreting cytokines and growth factors (Kim and Adamson, 2008). Macrophage secretory products, IL-8 and TNF-α, are known to enhance proliferation and adhesion of endometrial cells (Arici *et al*., 1998; Garcia-Velasco and Arici, 1999; Harada *et al*., 1999).

Inflammation plays an important role in the development of endometriosis. A large number of proinflammatory molecules have been implicated in the etiopathogenesis of endometriosis, such as TNF, IL-1, IL-8 and ICAM-1 (Olive and Pritts, 2001). The administration of anti-TNF antibody was an effective treatment of endometriosis in baboons (Agic *et al*., 2006).

Many studies have shown that COX-2 selective inhibition was effective in animal models for the treatment of endometriosis (Dogan *et al.,* 2004, Ozawa *et al*., 2006). In a rat model, treatment with the selective COX-2 inhibitor rofecoxib effectively caused regression of endometriotic explants. The effect was similar to that obtained with GnRH agonist leuprolide acetate, which is known to be one of the most effective drugs used in the medical treatment of endometriosis (Dogan *et al.,* 2004). Furthermore, a placebo-controlled double blind study has showed that rofecoxib was an effective, safe and inexpensive therapeutic

approach to endometriosis-associated pelvic pain in stages I and II endometriosis (Cobellis *et al*., 2004). In another study, Ozawa *et al*. (2006) investigated the effect of a selective COX-2 inhibitor (NS398) on lesion development in a severe combined immunodeficient (SCID)-xenograft model and reported a significant decrease in lesion size. However, according to Matsuzaki *et al*. (2004), COX-2 selective inhibition was more effective in prevention of ectopic implants in a rat model of endometriosis. Also, non-steroidal anti-inflammatory drugs (NSAIDs) (e.g. celexoxib, indomethacin, ibuprofen) decreased lesion burden in an autologous murine model (Efstathiou *et al*., 2005).

Increasing evidence supports the involvement of allergic-type inflammation (a hypersensitivity reaction) in endometriosis (Sugamata *et al*. 2005). In ectopic implants in endometriosis patients, diffuse infiltration of numerous mast cells was observed throughout the stromal lesions. These mast cells exhibited degranulation, and scattered granules were also observed. In the eutopic endometrium and normal uterine serosa of both the endometriosis patients and the controls, mast cells were rarely detected (Sugamata *et al*., 2005). Furthermore, it has been suggested that the presence of activated and degranulating mast cells within the nerve structures can contribute to the development of pain and hyperalgesia by direct effects on primary nociceptive neurons. Therefore, treatments targeting endometrial mast cells may prove effective in preventing or alleviating endometriosis-associated symptoms (D'Cruz, and Uckun, 2007).

Further evidence that allergic-type inflammation plays an important role in the occurrence and progression of lesions in endometriosis comes from animal studies. Using an experimental rat endometriosis model developed by uterine autotransplantation, Uchiide *et*

al. (2002) found that a hypersensitivity reaction was located in the lesional area. It is surprising that an allergic reaction involving mast cell degranulation was induced even by autologous transplantation of endometrial tissue (Umezawa *et al*., 2008). Moreover, Ihara *et al*., (2004) demonstrated that using a leukotriene (LT) antagonist having antiallergic action was able to relieve the endometriotic lesion in the rat endometriosis model. This further confirms that hypersensitivity enables the progression of the rat endometriosis lesion.

1.6.2. Positive-Feedback Model (PFM) as a Pathogenic Mechanism for Endometriosis

In 2005, Bulun *et al*. suggested a positive-feed back model (PFM) for the pathogenesis of endometriosis. In this model, high levels of local estradiol and prostaglandin E_2 (PGE_2) are maintained in endometriotic tissue by autoregulatory positive-feedback mechanism (Figure 3). Cytokines, angiogenic factors, PGE_2 itself, and estradiol all induce cyclo-oxygenase-2 (COX-2) expression and thereby ensure production of large quantities of PGE_2. Locally produced PGE_2, a potent stimulator of aromatase, upregulates estrogen production, which in turn stimulates COX-2 to increase PGE_2 leading to increased endometrial cell proliferation in endometriosis (Attar and Bulun, 2006, Bulun *et al*., 2005). Estrogen receptor β mediates the induction of COX-2 by estradiol in endometriotic tissue (Bulun, 2009).

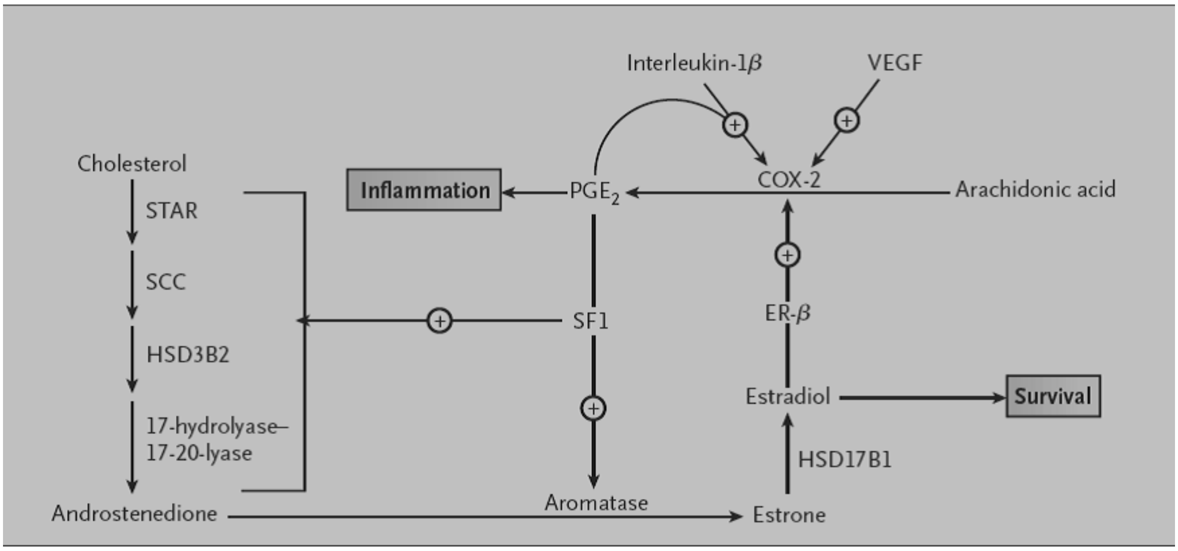


Figure 3. Positive-feedback model (PFM) as a pathogenic mechanism for endometriosis. This feed back loop involves nuclear receptors (steroidogenic factor 1 [SF1] and estrogen receptor β [ER-β]), enzymatic pathways, cytokines, and growth factors. COX-2 denotes cyclooxygenase-2, HSD17B1 17β-hydroxysteroid dehydrogenase 1, HSD3B2 3β-hydroxysteroid dehydrogenase 2, SCC side-chain cleavage enzyme, STAR steroidogenic acute regulatory protein, and VEGF vascular endothelial growth factor (Bulun, 2009).

1.6.3. NF-κB as a Major Culprit in an Autoregulatory Loop Model

Nuclear factor-κB (NF-κB) is a family of structurally related dimeric transcription factors that promote the expression of more than 150 genes involved in an array of cellular processes (Kumar *et al.*, 2004). Currently, it has five members, p50 (NF-κB 1), p52 (NF-κB 2), p65 (Rel A), c-Rel, and Rel B, which form various homo- and heterodimers, with the p50–p65 dimer being the most common (Ghosh *et al.*, 1998). In most cell types, inactive NF-κB complexes reside in the cytoplasm via their non-covalent interaction with inhibitory proteins known as IκBs. NF-κB activation requires degradation of its natural inhibitor (IκB), which depends on IκB phosphorylation by the IκB kinase complex (Barnes and Karin, 1997). The phosphorylation IκBs is mediated by a multimeric complex called IκB kinase (IKK) complex and its subsequent ubiquitination and degradation by the

proteasome. Thus, liberated NF-kB dimers capable of binding to DNA, translocate to the nucleus and activate the transcription of several target genes (Gonzalez-Ramos *et al*., 2007).

Many different signal transduction pathways originating from a wide variety of inducing mechanisms are able to converge on a single NF-κB/IκB complex. In addition, a wide variety of target genes are regulated by NF-κB, including genes involved in immune and inflammatory response, cell adhesion, cellular proliferation, and the anti-apoptotic response (Guo, 2007). NF-κB regulates the expression of certain genes, such as matrix metalloproteinase 9, tissue plasminogen activator and intercellular adhesion molecule 1 (ICAM-1) (Matsuzaki *et al*., 2005). ICAM-1 is an adhesion molecule expressed on the surface membrane of different cell types at sites of inflammation, which has been implicated in the development of endometriosis and is thought to play an important role in physiopathological processes in endometrial tissue (Guo, 2007).

NF-κB also acts upon the control of the cell cycle, which is critical in determining the degree of cellular apoptosis and proliferation (Van Langendonckt *et al*., 2002). Cell proliferation is a fundamental process in the development of endometriotic lesions (Carlberg *et al*., 2000). Induction of NF-κB by tumor necrosis factor (TNF) or other stimuli leads to the transcriptional activation of genes encoding proteins that act at different levels in the caspase cascade to suppress apoptosis (Matsuzaki *et al*., 2005). A resistance to apoptosis in regurgitated endometrial cells has been postulated to contribute to the development of endometriotic lesions (Tsai *et al*., 2001).

Several lines of evidence suggest that NF-κB could promote and maintain endometriosis (González-Ramos *et al*., 2008). In endometriotic cells, NF-κB appears to be constitutively activated, and suppression of NF-κB activity suppresses proliferation *in vitro* (Sakamoto *et al*., 2003) and *in vivo* (González-Ramos *et al*., 2008). Interestingly, all existing and nearly all investigational medications for endometriosis appear to act through suppression of NF-κB activation. Figure 4 summarizes the role played by NF-κB, as a major culprit, in an autoregulatory loop model for the pahogenesis of endometriosis. This model was proposed by Guo in 2007.

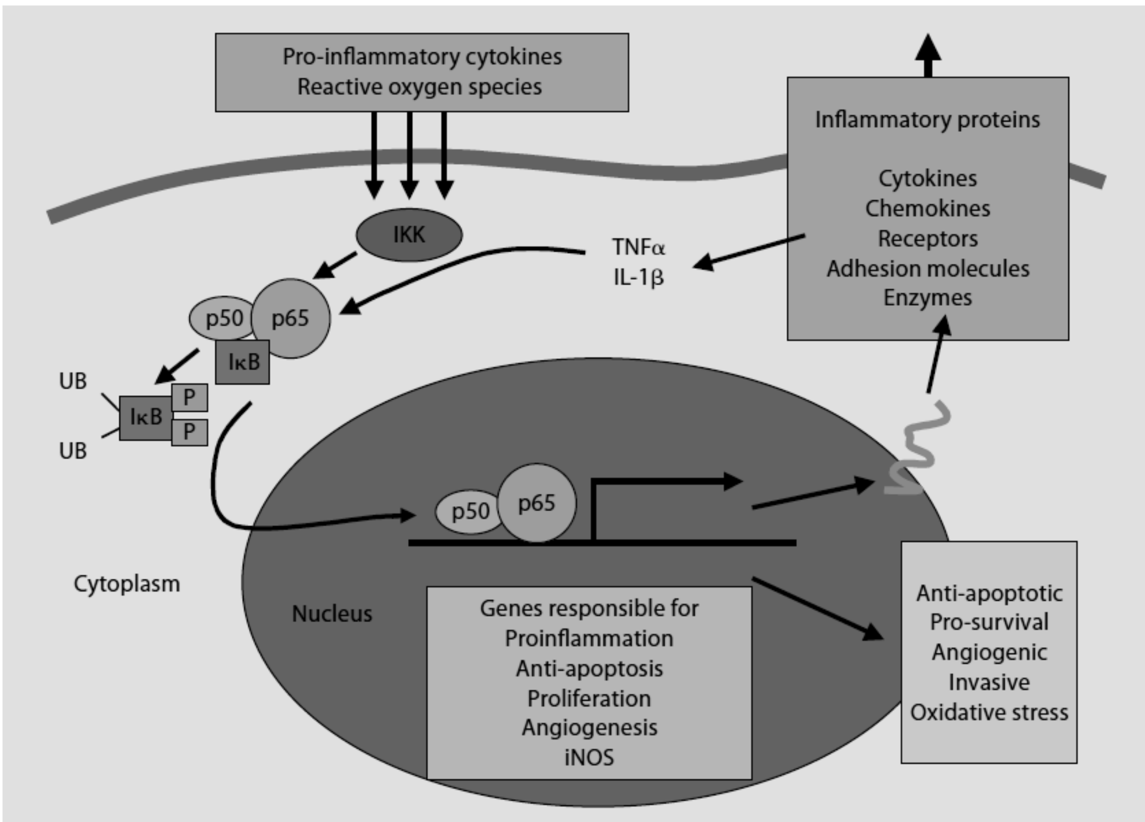


Figure 4. NF-κB as a major culprit in an autoregulatory loop model. Various stimuli activate NF-κB, which transactivates an array of NF-κB-target genes, which, in turn, further faciliate the production of pro-inflammatory cytokines and other stimulating factors, amplifying and perpetuating the loop. UB = Ubiquitination (Guo, 2007).

1.7. Aim of the Study

Endometriosis is a benign disease. However, it is in most cases a progressive one (Finas *et al*., 2008). In fact, it is not considered a life-threatening, but a life-altering disease that necessates continuous follow up and treatment. Until present, the diagnosis of endometriosis is only made by invasive tests (laparoscopy) (Kim and Adamson, 2008).

The treatment of endometriosis depends on temporarily removing the disease via surgical excision or temporarily suppressing it through medical therapy (D'Hooghe *et al*., 2009). Removal of lesion by surgical methods (laparoscopy or laparotomy) is far from ideal due to implied risks associated with all surgical interventions (Eltern, 2008). Since this disease is chronic and often relapses, long term or repeated courses of medical treatment are sometimes required. Efficacy of treatment is high during therapy. However, recurrence of symptoms occurs during treatment-free intervals (Panay, 2008).

Medical therapy includes estrogen–progestin combinations, progestogens, androgens, and gonadotropin-releasing hormone (GnRH) analogues. In general, these agents suppress the ovarian steroids and create a hypoestrogenic state to induce temporary atrophy of endometriotic lesions (Yao *et al*., 2005a). Therefore, medical therapy for endometriosis is rarely given to women wishing to conceive because treatment may further compromise fertility in these women (Panay, 2008).

Currently available medical therapies for endometriosis are unsatisfactory because they focus on treating the symptoms rather than curing the causes. In addition, they cannot be

used for prolonged duration because of severe secondary side effects (Rice, 2002). Thus, there is a definite need to develop new drugs to provide more efficient therapeutic alternatives that permenantly eradicate endometriotic lesions and prevent recurrence (Grümmer, 2006).

This work aims at searching for new therapeutic agents for endometriosis. The approach followed in this study was to focus on current understanding of the pathogenic mechanisms of the disease especially the role of the immune system and the similarity between endometriosis and cancer. Nine compounds were selected for this purpose. Some of which were natural products, others were drugs used to treat other conditions. The selected compounds can be categorized into the following groups:

1. Immunomodulators and anti-inflammatory agents: Including caryophyllene, citronellol, vitamin D, theophylline and the newly proposed selective estrogen receptor beta *in silico* hit NDGA (masoprocol) mentioned in Taha *et al*. (2010).

2. Some anti-angiogenic agents including caffeine.

2. Compounds with anti-cancer activity including: thymoquinone, sunitinib, a well known protein tyrosine kinase inhibitor, and primaquine which inhibits heat shock protein 90 (Sheikha *et al*., 2010).

Literature Review

2.1. Angiogenesis and Vasculogenesis in Endometriotic Lesions

A typical feature of endometriotic lesions is their dense vascularization. Early lesions, which are the most active ones, appear as pink-red in color due to their high vascularization compared to black lesions. Moreover, the areas surrounding the lesions also show an increased vascularization (Laschke and Menger, 2007).

Experimentally, it has been demonstrated that the establishment of an effective blood supply is essential for the survival of endometrial implants and the development of endometriosis (Taylor *et al.*, 2002). This neovasularization provides the nutrients and growth factors and promotes the recruitment of inflammatory cells (Van Langendonckt *et al.*, 2008).

The development of new blood vessels occurs via two principal processes namely angiogenesis and vasculogenesis (Van Langendonckt *et al.*, 2008). Angiogenesis is defined as the formation of new microvessels from pre-existing ones. Physiological angiogenesis is essential for embryogenesis. However, in adults angiogenesis rarely occurs except in reproductive tissues and wound healing. Profound non-pathological angiogenesis is observed in the ovary during corpus luteum formation and in the endometrium during menstrual cycle (Smith, 1998).

Vasculogenesis was originally defined as *de novo* blood vessel formation by migration and differentiation of angioblastic progenitor cells during embryonic and fetal development (Kabmeyer *et al.*, 2009). However, it has been recently demonstrated that vasculogenesis

also contributes to the formation of new blood vessels in the adult (Shi *et al*., 1994; Asahara *et al*., 1997). This post-natal vasculogenesis is characterized by the mobilization of bone marrow-derived or tissue-resident endothelial progenitor cells (EPCs) into the blood stream in response to certain cytokines or tissue ischaemia. EPCs are then recruited into sites of neovascularization, where they are incorporated into the vascular endothelial lining and differentiate *in situ* into endothelial cells (Murasawa and Asahara, 2005).

A recent study conducted by Becker *et al*. (2011) has shown that both vasculogenesis and sprouting angiogenesis occur at the same time in endometriotic lesions. Post-natal vasculogenesis is characterized by the recruitment of circulating endothelial progenitor cells (EPCs) from the bone marrow (Figure 5). At sites of hypoxia, they attach, proliferate and are finally incorporated into the endothelium, where they differentiate into mature endothelial cells (Laschke *et al*., 2011). In fact, in a murine model of surgically induced endometriosis, up to 37% of all endothelial cells in endometriotic lesions were bone marrow-derived one week after transplantation.

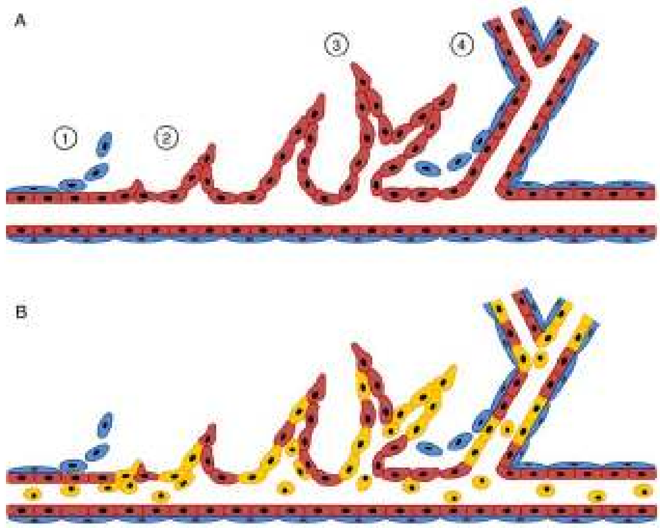


Figure 5. Mechanisms of blood vessel formation during endometriosis. (A) Sprouting angiogenesis: perivascular cells, such as pericytes and smooth muscle cells, (blue), endothelial cells (red); (B) Post-natal vasculogenesis and sprouting angiogenesis occur in parallel in endometriotic lesions. Circulating EPCs (orange) (Laschke *et al*., 2011).

In peritoneal fluid from endometriosis patients there is an imbalance between pro- and antiangiogenic growth factors (Laschke and Menger, 2007). The level of vascular endothelial growth factor (VEGF) is elevated in the peritoneal fluid from women with endometriosis (Bourlev *et al*., 2006). In fact, inflammatory cells such as activated macrophages produce proangiogenic factors, contributing to this proangiogenic environment (Gazvani and Templeton, 2002). Interestingly, it has been demonstrated that dendritic cells have a role in supporting angiogenesis in endometriosis in a murine model of the disease (Fainaru *et al*., 2008). It has been suggested that suppression of blood vessel development by inhibition of specific angiogenic factors is a possible novel therapeutic opportunity in the treatment of endometriosis (Laschke *et al*., 2006). Blocking the angiogenic cascade impairs endometriosis-like lesion development, as shown in various

animal models (Hull *et al*., 2003; Laschke *et al*., 2006) and in the chicken chorioallantoic membrane (Nap *et al*., 2005). Angiostatic therapy can be directed at various elements of the angiogenic response, i.e. activation, proliferation, adhesion, migration and maturation of endothelial cells (McCarty *et al*., 2003).

Several factors must be taken into consideration with respect to the use anti-angiogenic agents in endometriosis treatment. First, anti-angiogenic agents may only be useful for early stage disease as they may not effectively target preexisting pericyte-protected vessels. Second, chronic administration of these drugs is required and third, the potential adverse effects on other processes such as reproductive function must be taken into consideration (Nothnick and Zhang, 2009).

2.2. Apoptosis in Endometriosis

Apoptosis is a physiologic process that eradicates undesired cells without eliciting an inflammatory reaction. It is a distinctive form of programmed cell death which is defined by characteristic morphological and biochemical events resulting in the efficient elimination of cells from tissue (Agic *et al*., 2009). Morphological alterations in apoptosis include: Membrane blebbing, aggregation of chromatin at the nuclear membrane, shrinking of cytoplasm, condensation of nucleus, fragmentation of cell into smaller bodies, formation of apoptotic bodies and pore formation in mitochondrial membrane involving proteins of the Bcl-2 family. Biochemical features of apoptosis include: Energy (ATP) requirement, release of cytochrome c, apoptosis-inducing factor (AIF) and other factors into cytoplasm by mitochondria, activation of caspase cascade, alterations in membrane biochemistry (i.e.

translocation of phosphatidylserine from the cytoplasmic to the extracellular side of the membrane) and presence of ladder pattern of DNA fragmentation after agarose gel electrophoresis (Harada *et al*., 2004).

Apoptosis plays an important regulatory role in eutopic endometrium in maintaining cellular homeostasis during the menstrual cycle through the elimination of aging cells from the functional layer of the uterine endometrium. Electron micrographs revealed the presence of apoptotic bodies in human endometrial epithelial cells during the late secretory phase (Otsuki *et al*., 1994). Similarly, apoptotic cell death was reported in the uterus during estrous cycle in laboratory animals. An inverse correlation between cell death and cell proliferation existed in uterine epithelial cells during the estrous cycle in rats. In uterine luminal and glandular epithelial cells, apoptotic index showed peaks at metestrus and estrus, respectively (Figure 6) (Sato *et al*., 1997).

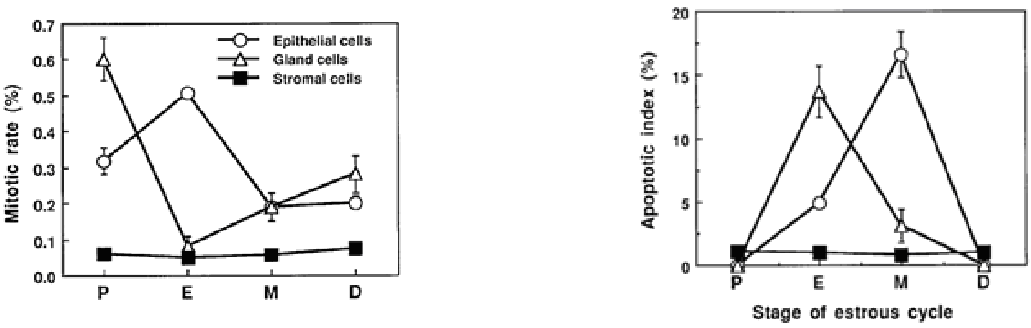


Figure 6. Mitotic rate and apoptotic index in rat uterus during the estrous cycle. P, proestrus; E, estrus; M, metestrus; D, diestrus (Sato *et al*., 1997).

It has been suggested that endometriosis could result from increased cellular proliferation or decreased apoptosis in response to appropriate stimuli (Agic *et al*., 2009). In fact, accumulating evidence suggests that endometrial cells from women with and from those without endometriosis have fundamental differences. These include enhanced proliferation (Johnson *et al*., 2005) and an increased ability to implant and survive in ectopic locations (Meresman *et al*., 2000). Furthermore, it has been demonstrated that the apoptotic index in glandular epithelium was significantly lower in women with endometriosis than in controls (Dmowski *et al*., 2001). Therefore, impaired sensitivity of endometrial tissue to spontaneous apoptosis contributes to the abnormal implantation and growth of endometrium at ectopic sites.

Reflux of endometrial fragments during menstruation into the peritoneal cavity is a common phenomenon. Normally, cells which do not adhere to their extracellular matrix enter apoptosis as they receive different signals from their adhesion receptors (Aplin *et al*., 1998). However, in endometriosis, these cells have the ability to adhere to mesothelial cells of the peritoneum, proliferate and produce neoangiogenesis resulting in the development of active endometriosis implants (Agic *et al*., 2009).

Recent studies from (González-Ramos *et al*., 2007; González-Ramos *et al*., 2008) showed constitutive nuclear factor-κB (NF-κB) activation in peritoneal endometriosis. They reported that the NF-κB pathway is implicated in the development of endometriotic lesions *in vivo*. In an animal model NF-κB inhibition reduced intracellular adhesion molecule-1 expression and cell proliferation and increased apoptosis of endometriotic lesions diminishing the initial development of endometriosis.

In endometriotic tissues, genes related to apoptosis (GADD34, GADD45A, GADD45B, PIG11) were downregulated (Arimoto *et al*., 2003). Increased expression of anti-apoptotic factors (Bcl-2) and decreased expression of pre-apoptotic factors (BAX) was reported (Meresman *et al*., 2000). Thus, dysregulation in expression of pro- and anti-apoptotic regulatory genes characterized eutopic and ectopic endometrial cells from women with endometriosis.

The development of endometriosis and its persistence requires either or both inherent resistance of the endometriotic cells and/or impaired capacity of the immune cells to mediate endometriotic cell killing. Both mechanisms appear to be operative in endometriosis (Dmowski and Braun, 2004). In endometriosis, the number and secretory activity of peritoneal macrophages are increased and recent evidence suggests that these cells play an important role in the development and maintenance of endometriosis (McLaren *et al.*, 1997). Women with endometriosis had a significantly higher proportion of Bcl-2 positive macrophages than the non-endometriotic group. On the other hand, the proportion of Bax positive peritoneal fluid macrophages was significantly elevated in women without endometriosis. The increased proportion of Bcl-2 positive macrophages may predispose them to resist apoptosis which may have important consequences on the survival of the ectopic endometrial tissue (McLaren *et al.*, 1997).

2.3. Molecular Similarities between Endometriosis and Cancer

Despite being a benign disorder, recent studies suggest that endometriosis could be viewed as a neoplastic process (Varma *et al*., 2004). Evidence to support this hypothesis comes from the finding that some tumors, especially endometrioid and clear-cell carcinomas, can arise from endometriosis in humans (Gorp *et al*., 2004).

A very strong support for the role of tumor suppressor phosphatase and tensin homolog (PTEN) gene in the malignant transformation of endometriosis has been demonstrated recently from a mutagenesis in genetically engineered mice. An elegant experiment conducted by Dinulescu *et al*. in 2005 has shown that forced expression of oncogenic K-*ras* or conditional PTEN gene deletion results in endometrial growths. However, the combination of both gives rise to carcinoma, indicating the potential relevance of these genetic alterations to the malignant transformation of endometriosis. So, this finding is consistent with an increased risk of ovarian cancer in women with endometriosis (Matzuk and Lamb, 2008).

Molecular similarities between endometriosis and cancer do exist (Varma *et al*., 2004). These similarities include:

I- Self-sufficiency in growth signals: endometriosis is an estrogen-dependent neoplasm. Several factors contribute to this estrogen dependency including: 1) Increased local production of estrogen through increased expression of aromatase cytochrome P450 (Bulun *et al*. 2000). 2). Increased responsiveness to estrogen through increased ER-α expression in

active lesions (Matsuzaki *et al*. 2001). 3) Inherited genetic polymorphisms in estrogen and progesterone receptors (Kitawaki *et al*. 2001).

II- Insensitivity to anti-proliferative signals: endometriosis may resist the antiproliferative effect of progesterone by the predominant expression of the inhibitory progesterone receptot (PR-A) isoform instead of the stimulatory PR-B isoform (Attia *et al*. 2000).

III- Resistance to apoptosis: Malignant tumors usually over express of antiapoptotic (Bcl-2) and under-express of pro-apoptotic (BAX) factors. Similarly, endometriotic lesions evade apoptosis through increased Bcl-2 and decreased BAX (Meresman *et al*. 2000).

IV- Sustained angiogenesis: pathological angiogenesis exists in both endometriosis and cancer processes in which immune cell suppression and immune cell activation co-exist (Folkman 2002, Gazvani and Templeton 2002).

V- Tissue invasion and metastasis: In invasive cancers degradation of the basement membrane and surrounding stroma occurs. To do so, tumors secrete proteases (e.g. MMPs). In fact, there is correlation between the expression of MMP-2 and MMP-9 and with the grade and stage of many cancers (Varma *et al*., 2004). According to Borghese *et al*. (2008), deep endometriosis is able to invade the surrounding tissues. Furthermore, it sometimes metastasizes in lymph nodes and beyond the abdominal cavity. Interestingly, MMP activity is up-regulated in endometriotic lesions (Mizumoto *et al*. 2002). However, in endometriosis, the invasion is controlled. In other words, it stops at a given time. This

explains why endometriosis is not a fatal disease. However, it has a unique feature of being a benign metastatic disease (Varma *et al*., 2004).

VI- Genomic instability: Among the studied features in endometriosis: 1) Gain in oncogenic activity 2) inactivation of tumour suppressor gene (TSGs) (genes that encode for proteins which inhibit excess cellular proliferation and malignant transformation) and 3) Abnormalities in DNA mismatch repair enzymes (Varma *et al*., 2004).

In summary, clinicopathological, molecular and genetic evidences support the hypothesis that endometriosis is a neoplastic process with a potential for malignant transformation. In fact, endometriosis and cancer have close but distinct features in common. However, some control points prevent endometriotic cells from getting a more aggressive phenotype (Borghese *et al*., 2008).

2.4. Medical Therapies

Treatment in endometriosis aims at alleviating pain, preventing progression and recurrence of the disease, and restoring or preserving fertility (Van Langendonckt *et al*., 2008). Current therapies attempt to relieve symptoms through surgical and/or medical treatment. Surgical procedures are far from ideal due to implied risks associated with surgical interventions (Eltern, 2008). Furthermore, cumulative recurrence rates after conservative surgery can be as high as 50–70% (Fedele *et al*., 1994).

2.4.1. Analgesics

Few randomized controlled trials evaluating the effect of analgesics, such as NSAID, in treating endometriosis associated pain exist. Despite this, NSAID are often used in women with endometriosis with variable outcomes (Panya, 2008).

2.4. 2. Ovulation/Estrogen Suppression

Both laboratory and clinical studies demonstrated that most endometriotic lesions contain estradiol, progesterone, and androgen receptors. It is well known that estradiol promotes growth in endometriotic lesions. On the other hand, androgens cause atrophy. Progestins cause complex effects depending on the dose and progestin used (Rice, 2002).

The first drug approved for endometriosis by the Food and Drug Administrattion (FDA) was danazol (Dmowski *et al.*, 1971). Danazol (Danol) is a synthetic steroid chemically related to 17-ethinyl testosterone. It suppresses the hypothalamic-pituitary-ovarian axis by inhibiting the output of gonadotrophins from the pituitary. In addition, it may act by inhibiting binding of sex steroids to cellular receptors in target tissues and possibly by decreasing the synthesis of sex steroids. It has, therefore, been used to reduce hormonal stimulation to the endometrium in endometriosis (Farquhar *et al.*, 2007; Paterson and Feely, 1983). Although effective for endometriosis, danazol use is limited by its masculinizing side-effects. These androgenic and anabolic side effects include decreased breast size, atrophic vaginitis, hot flushes, emotional lability, vaginal dryness, changes in libido, weight gain, oedema, muscle cramps, acne, oily skin, sweating, deepening of voice, hirsutism, nausea and abnormal bleeding pattern (Farquhar *et al.*, 2007).

Other commonly used therapeutic agents for endometriosis are GnRH agonists, which effectively repress the hypothalamic-pituitary-ovary axis. Binding of these drugs to GnRH receptors result in stimulation of gonadotropin release. In fact, GnRH analogues have a remarkably longer half life than natural GnRH. Consequently, the pituitary is exposed to continuous GnRH stimulation. This results in downregulation of its function and reduction of LH and FSH secretion. So, after this initial increase in gonadotropin secretion (a flare), cessation follows. Therefore, secretion of ovarian steroids like estradiol is blocked (Eltern, 2008). In most cases, pain relief is achieved after using GnRH analogues. However, side effects due to the hypoestrogenic status exist that range from dizziness, vaginal dryness, loss of libido to increased risk of thrombosis and osteoporosis (Griesinger *et al.*, 2005). "Add back" hormone replacement therapy is given when GnRH analogue treatment was extended longer than six months to provide protection against osteoporosis (Garai *et al.*, 2006). However, bone density should be monitored with long-term usage (Panya, 2008).

Contraceptive pill was effective in treating endometriosis-related pain. Progestins (e.g. oral or depot medroxyprogesterone acetate) at high doses were used to suppress the cycle, (Table 1). However, they produce premenstrual syndrome-type effects and irregular bleeding (Panya, 2008). A frequently applied therapy for endometriosis is combined oral contraceptives (COCs). The use of COCs leads to a pregnancy-pretending hormonal state with decreased gonadotropin levels, ovulation inhibition and reduced menstrual flow (Rice, 2002). In fact, COCs are widely utilized as initial therapy, often together with analgesic non-steroidal anti-inflammatory drugs (NSAIDs). This approach is accompanied by fewer side effects, but like other anti-hormonal treatments, eliminates the possibility for the female to get pregnant (Eltern, 2008).

Table 1. Hormonal medications used to treat endometriosis (Rice, 2002).

Category	Drug
Androgen	Danazol
GnRH agonist	Leuprolide
	Leuprolide depot
	Buserelin
	Goserelin
	Nafarelin
Progestins- chemical variants of natural progesterone.	Gestrinone
	Medroxyprogesterone acetate
Oral contraceptives	Combination estrogen/ Progestin

In summary, current hormonal medications used to treat endometriosis produce a hypo-estrogenic state thereby causing lesion regression. However, this hypo-estrogenism produces side-effects such as decreased bone density (D'Antonio *et al*. 2000). So, it was suggested that a combination of surgical and medical therapies might provide better outcomes than any one option alone (Rice, 2002).

New therapeutic agents for endometriosis have been developed based on the current understanding of the pathophysiology of endometriosis. The new drugs (Table 2) either specifically targeting endometrial lesions or modulate the immune system. The former group includes selective estrogen and progesterone receptor modulators, ligands and vascular growth factor inhibitors (Panay, 2008).

Table 2. Medical interventions for the treatment of endometriosis (Panay, 2008).

Analgesia/anti-inflammatory Agents	**Suppression of ovulation/estrogen**	**Direct action in endometriotic deposits**	**Immunomodulation**
NSAIDS*	Contraceptive pill*	LNG IUS*	Inflammatory modulators***
	Danazol*	Progesterone antagonists**	
	Gestrinone*	SPRMs***	
	GnRH agonists*	SERMs**	
	Aromatase inhibitors (AIs)*	AIs* (estrogen suppression)	
		ER ligands***	
		Angiogenesis inhibitors**	
		Statins**	

*Currently available and sufficient evidence to recommend usage.
**Currently available but insufficient evidence to recommend usage.
***Product(s) in development (basic science or phase I, II, III trials).

2.4.3. Drugs with Direct Action on Endometrial Deposits

2.4.3.1. Levonorgestrel Intrauterine System

For over a decade, the levonorgestrel intrauterine system (LNG IUS) has been used for contraception. In addition, it has been suggested that the steady low-level release of levonorgestrel have a direct effect on endometriotic deposits through peritoneal fluid. Evidence exists from pilot studies on levonorgestrel which have shown a great improvement in pain control in women with endometriosis confirmed by prior laparoscopy. Furthermore, a reduction in ultrasonographic size of rectovaginal nodules was evident as well as an improvement in the American Fertility Society (AFS) staging of the disease at second look laparoscopy (Panay, 2008).

2.4.3.2. Aromatase Inhibitors

Estrogen is the most important known factor that stimulates the growth of endometriosis. It was thought that estrogen delivery to endometriotic implants is by the circulating blood in an endocrine fashion. However, an autocrine positive feedback mechanism was discovered which favored the continuous production of estrogen and prostaglandin (PG)E_2 in the endometriotic stromal cells. Aromatase, the enzyme that catalyzes the conversion of C_{19} steroids to estrogens, is expressed in endometriotic stromal cells. Thus, locally produced estrogen stimulates cyclooxygenase-2 (COX-2) to increase the levels of PG E_2. PG E_2, in turn, is a potent inducer of aromatase activity in endometriotic stromal cells (Bulun *et al.*, 1999).

Aromatase inhibitors, primarily used in the treatment of breast cancer, have emerged as a major means of treating endometriosis (Nothnick and Zhang, 2009). These agents induce regression of endometriosis by disrupting local estradiol production by the endometriotic implant. The first reported use of an aromatase inhibitor to treat endometriosis in humans was in 1998 by Takayama. Later on, potent and selective third-generation non-steroidal aromatase inhibitors become available. Anastrozole and letrozole have substantial advantages over earlier agents in terms of efficacy and tolerability (Ferrero *et al.*, 2009). Anastrozole or letrozole combined with standard regimes have been used successfully to alleviate chronic pelvic pain and reduce the lesion size (Attar and Bulun, 2006). However, in some cases aromatase inhibitors led to ovarian cysts and in other cases recurrence happened during follow-ups after the treatment. So, it appears that the use of aromatase

inhibitors has to be individualized by carefully choosing the combined medical treatments to minimize side-effects and prevent recurrences (Nothnick and Zhang, 2009).

Irreversible steroidal aromatase inactivators (such as exemestane) have been successfully used in breast cancer patients. However, the permanent inhibition of aromatase activity is contraindicated in women with endometriosis wishing to preserve their fertility (Ferrero *et al*., 2009).

2.4.3.3. Progesterone Antagonists

Progesterone antagonists such as mifepristone (RU486) have been shown to block progesterone receptors in endometriotic tissue (Panay, 2008). In surgically induced endometriosis in rats, onapristone (ZK 98 299) induced a 40–50% reduction of lesions while ZK 136 799 induced a 63–75% reduction (Stoeckemann *et al*., 1995). In monkeys, mifepristone reduced surgically induced endometriotic lesions after 9 months of treatment. In a clinical trial, there was an improvement in symptoms in all treated patients treated with mifepristone and a 55% mean regression of visible endometriosis after 6 months of treatment following the 50 mg dose (Chabbert-Buffet *et al*., 2005).

2.4.3.4. Selective Estrogen Receptor Modulators

Selective estrogen receptor modulators (SERMs) have an antiproliferative effect on endometriotic tissue. In surgically induced endometriosis model in the rat, significant regression of endometriotic implants was achieved using raloxifene after 14 days of

treatment (Yao *et al*., 2005a). Similarly, raloxifene was effective in treating spontaneous endometriosis in mature female rhesus macaques (Fanning *et al.*, 1996).

In 2005, a clinical trial on the use of raloxifene to treat endometriosis was terminated early due to unfavorable outcome (Guo and Olive, 2007). In another study conducted in 2008, chronic pelvic pain from endometriosis returned after raloxifene treatment (Stratton *et al*., 2008). The two apparently unsuccessful clinical trials on endometriosis indicate that there is a need to better understanding of this disease through basic research. Also, human endometriosis trials may have different outcome compared to animal studies since anatomy and physiology of the two species are often divergent. Furthermore, SERMs are often tissue and cell-specific in their action. So, they should not be simply grouped together nor should extrapolations made from one compound to another (Guo and Olive, 2007).

2.4.3.5. Selective Progesterone Receptor Modulators

Selective progesterone receptor modulators (SPRMs) are a new class of progesterone receptor ligands. They have mixed agonist/antagonist effects on the progesterone receptors depending on the target tissue (Panay, 2008). Asoprisnil was the first SPRM to reach advanced stages of clinical development for the treatment of endometriosis and fibroids. In phase I and II clinical trials, there was a dose-dependent suppression of endometriosis-associated pain (Chwalisz *et al*., 2004). Unfortunately, the development of asoprisnil has currently stopped because of concerns regarding endometrial proliferation and hyperplasia in some subjects (Panay, 2008).

The advantage of SPRMs over the GnRH analogues is that SPRMs does not produce a hypo-estrogenic state. This indicates that the adverse effects of the GnRH analogues are avoided. Also, because of their selective nature, other progestogenic adverse effects are minimized (Panay, 2008).

2.4.3.6. Estrogen Receptor Ligands (or Selective Estrogen Receptor Beta Agonists)

Selective estrogen receptor beta (ERβ) agonists exhibit high selective binding to ER beta (200 times greater than alpha). In an experimentally induced model of endometriosis, the ERβ agonist ERβ-041 caused complete lesion regression in 40-75% of the animals (Harris *et al*., 2005). In a clinical trial, healthy cycling women received up to 150 mg daily doses of ERβ-L for 28 days had normal ovulatory reproductive hormone profiles and ovulation during three cycles and normal menses occurred at the end of each cycle. Preliminary phase II data indicated that ERβ-L reduced symptoms of endometriosis (Panay, 2008).

Estrogen receptor beta agonists, like ERβ-041, lack classic estrogenic activity in several *in vivo* model systems (Harris *et al*., 2003). So, they have the advantage over GnRH agonists, which suppress the hypothalamic– pituitary–gonadal axis (Couse and Korach, 1999). Also, ERβ regulates few if any genes in normal mice. So, it is expected that ERβ agonists will have a favorable side-effect profile (Harris *et al*., 2005).

2.4.3.7. Antiangiogenic Agents

Several antiangiogenic agents were effective for the treatment of endometriosis as recently reviewed by Becker and D'Amato (2007). These agents include VEGF-A- blocking antibodies and flt-1 decoy receptor (Hull *et al*., 2003), endostatin (Becker *et al*., 2005, 2006; Nap *et al*., 2004), the antiangiogenic agent TNP-470 (Nap *et al*., 2004), a non-toxic form of TNP-470 (Becker *et al*., 2006), anginex (Nap *et al.*, 2004), 2- methoxyestradiol (Becker *et al*., 2008) and selective cyclo-oxygenase inhibitors (Matsuzaki *et al*., 2004).

Anti-angiogenic agents display different mode of action. They can affect pro-angiogenic cytokines and inhibit their interaction with their receptor (e.g. VEGF-A inhibitors) or have a direct inhibitory impact on endothelial cells (Tabruyn and Griffioen, 2007). Agents affecting pro-angiogenic cytokines include a soluble form of truncated VEGF receptor (sFlt-1) or an affinity-purified antibody against VEGF. These two agents significantly inhibited endometriotic lesion growth in a mouse model of endometriosis (Hull *et al.*, 2003; Nap *et al.*, 2004).

The 3-hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase inhibitors, referred to as statins, are used widely for lowering cholesterol synthesis by blocking conversion of HMG-CoA to mevalonate. Statins have been shown to exhibit anti-inflammatory and anti-angiogenic activity. Esfandiari *et al*. (2007) has reported that the effect of statins on angiogenesis is biphasic and dose-dependent. Low statin doses induce a proangiogenic effect, whereas high statin doses have anti-angiogenic effect. Treatment of induced endometriosis in mice with endostatin, a proteolytic cleavage product from collagen XVIII,

inhibited the growth of newly formed lesions. Additionally, no impact on fertility or teratogenic effects was found. However, endostatin had not affected the growth of the already established implants (Becker *et al.*, 2005).

Inhibitors of angiogenesis exhibited a preventive effect in endometriosis through the interferance with new vessel formation. However, there are limitations for their use since chronic administration is required. Furthermore, they cannot target mature vessels surrounded by pericytes indicating that they are likely to be of particular benefit in early stages of the disease or to prevent recurrence after surgery (Benjamin and Keshet, 1997). Unfortunately, when advanced endometriosis is diagnosed, it has usually been present for years. So, it has already established its pericyte-protected blood vessels (Groothuis *et al*., 2005). So, these vessels are resistant to conventional antiangiogenic therapy (Benjamin and Keshet, 1997; Nap *et al*., 2004).

Using the skinfold chamber model, Laschke *et al*. (2006) showed that combined inhibition of VEGF, fibroblast growth factor and PDGF was much more effective to prevent vascularization of endometrial grafts than VEGF treatment alone. So, combining agents inhibiting both angiogenesis and vessel maturation may prove more efficient in the treatment of endometriosis.

One of the main challenges of antiangiogenic therapies in endometriosis is to selectively target pathological angiogenesis, without altering physiological angiogenesis essential for reproductive function and wound healing. Several anticancer drugs with antiangiogenic potential have been found to have a harmful effect on reproductive function in humans

(Klauber *et al*., 1997). However, there is limited information about the possible side effects of antiangiogenic treatment in endometriosis (Laschke and Menger, 2007).

2.4.4. Immunomodulators/Inflammatory Modulators

The peritoneal immune system is impaired in endometriosis patients, particularly natural killer cell activity. Also, there are certain similarities between endometriosis and autoimmune disease (Panay, 2008). In light of these facts, many studies have demonstrated the beneficial effect of immunomodulators/ anti-inflammatory drugs on treating endometriosis (Altintas *et al*., 2008; D'Hooghe *et al*., 2006).

A study in baboons with spontaneous endometriosis showed that inhibition of TNF-α, which plays an essential role in the regulation of pro-inflammatory cytokines such as IL-1 and IL-6, reduces the number of existing red lesions (Barrier *et al*., 2004). In another study, D'Hooghe *et al*. (2006) found that tumour necrosis factor-alpha-binding protein (r-hTBP1) inhibits the development of endometriosis in rodent and baboon models of the disease. Although TNF-α blockers have a promising potential to treat endometriosis, there is concern about the possibility that anti-TNF-α therapies may negatively affect host defense, such as causing infections and malignancies. Subsequently, necessary measures should be taken (Ceyhan *et al*., 2011).

Velasco *et al*. (2007) reported a beneficial effect of a combined treatment with one dose of intracystic recombinant IL-2 and GnRH analogue in women with endometriosis after transvaginal puncture and aspiration of endometriomata. A decrease in patient serum

cytokine production was reported. Also, the time until the recurrence of the disease was significantly longer. In another clinical trial, patients treated with two doses of IL-2 had better clinical outcome than patients who received only one (Acién *et al*., 2005).

A randomized, double-blinded, placebo-controlled clinical trial involving 28 women with minimal and mild endometriosis showed that the group taking rofecoxib, a selective COX-2 inhibitor, for 6 months has significant improvement of both pelvic pain and dyspareunia as compared with the placebo group (Cobellis *et al*., 2004). Unfortunately, rofecoxib (Vioxx) and valdecoxib (Bextra) have been recently withdrawn from the market following studies illustrating that the use of rofecoxib increases the risk of serious coronary heart disease and so do other selective COX-2 inhibitors. This implies that the future of using selective COX-2 inhibitors to treat endometriosis is uncertain (Wu and Guo, 2007).

Ingelmo *et al*. (1999) examined the effect of the immunomodulatory agent interferon (IFN) on an experimental rat model of endometriosis. IFN caused shrinkage of endometrial implants in short regimens. It was suggested that the local antiproliferative effect of interferon developed more rapidly than its immunomodulatory effect (Itil *et al*., 2006). In another study, IFN-α-2b regressed significantly both the size and histological components of the endometriotic implants in rats (Altintas *et al*., 2008). Ali *et al*. (2000) administered laparoscopic intraperitoneal injection of IFN-α-2b to women with pelvic endometriosis. Twenty-five infertile women with stages II, III or IV disease were enrolled. At the three-month follow-up examination, all symptoms and signs had decreased significantly. The decrease was proportional to the size and diameter of large implants and to the degree of endometriosis. A possible explantation for these findings is that IFN-α-2b may activate

macrophages that help in the suppression of endometriosis growth (Altintas *et al*., 2008). On the other hand, Acien *et al*., (2002) administered IFN-α-2b in the pouch of Douglas at the end of laparotomic conservative surgery in infertile women with moderate or severe endometriosis. Unfortunately, the later recurrence of endometriosis was increased.

Ceyhan *et al*., (2011) studied the effect of two well-known immunomodulators, infliximab and etanercept, on the regression of endometriotic implants. Both compounds decreased implant size in experimental endometriosis without lowering VEGF expression. In another study using the potent immunomodulatory drug Loxoribine, regression in both stromal and epithelium components were obtained in a rat model of endometriosis. Interestingly, there were significantly greater numbers of dendritic cells in the explants of animals (keenen *et al*., 1999). Similarly, leflunomide, a disease-modifying immunomodulator with anti-inflammatory activity, significantly reduced the size of the endometrial explants in the rat model (Uygur *et al*., 2006).

Lipoxins (LX) are arachidonic metabolites that have been characterized as anti-inflammatory lipid mediators. Biological actions of lipoxins are mediated through specific receptors. It has been suggested that they offer a novel approach to achieve control of inflammation (Serhan and Savill, 2005). Tissues from patients with ectopic endometriosis showed an elevated expression of lipoxin receptors compared with the normal tissues. Therefore, it was hypothesized that they have great role in the development of endometriosis. Chen *et al*. (2009) demonstrated that 15-R-LXA4 was able to inhibit the progression of endometriosis in a murine model of the disease. It exerted its action mainly by suppressing the gene and protein expression of IL-1β and TNF-α.

Bacillus Calmette-Guerin (BCG) is used as a booster for the immune system. In clinical settings, it has been administered with tumor cells, to make the immune system more responsive to the antigens presented (Itil *et al*., 2006). Gul *et al.* (2001) investigated whether BCG vaccination could prevent endometrial implantation in the iris of the eye in a rat model of endometriosis. They concluded that systemic prophylaxis with BCG can produce an inhibitory effect on endometrial transplantation. In another study, Itil *et al*. (2006) found that BCG systemic prophylaxis exerts an inhibitory effect on peritoneal endometriotic implants in a rat model. Vaccination of rats with RESAN, a newly developed immunomodulator, was also effective in both the prophylaxis and treatment of endometriosis (Szymanowski *et al*., 2009).

2.5. Animal Models in Endometriosis Research

Several factors contribute to the lack of understanding of the patho-physiological mechanisms of endometriosis. Most importantly, at the time of clinical presentation most women have already established the disease. Furthermore, objective long-term studies in women are difficult to conduct because of individual differences in progression and symptomatology of the disease (Hastings and Fazleabas, 2006). Also, disease progression can only be confirmed by repeated laparoscopic examinations (Eltern, 2008). So, ethical and practical considerations limit studies in the human. Therefore, animal models are of great value in endometriosis research.

Endometriosis only affects naturally humans and non-human primates (Grummer, 2006). However, different animal models have been successfully developed by transplanting

endometrial tissue to ectopic sites. These animal models can be classified into two types, homologous and heterologous models. In homologous models, surgical transplantation of endometrium of the same or syngeneic animals in immunocompetent animals was performed as in Jones (1984) and Vernon and Wilson (1985). Alternatively, human endometrial fragments are transferred either intraperitoneally or subcutaneously to immunodeficient mice in heterologous models as in Zamah *et al*. (1984) or onto the chicken chorioallantoic membrane (CAM) as in Maas *et al*. (1999).

I-Primate Models

Endometrioisis occurs naturally in some non-human primates. Actually, it has been reported in 11 different monkey species (Story and Kennedy, 2004). Most experiments were conducted with Rhesus macaques and baboons. In these animals, spontaneous endometriosis develops slowly and with low frequency in the wild. However, its frequency increases in captivity (Hastings and Fazleabas, 2006). Artificial induction of endometriosis has been also established in non-human primates. Different methods were employed like repositioning of the cervix in the first experiments (Scott and Te-Linde, 1950), suturing of endometrium fragments at ectopic sites in the peritoneal cavity (Fazleabas *et al*., 2002) or injection of endometrium (D'Hooghe *et al*., 1995).

Although non-human primates are the most appropriate model to investigate endometriosis, the infrequent rate and the long periods to disease manifestation as well as high costs make this model impractical for systematic evaluation in endometriosis research. For these

reasons, small laboratory animal models, mainly performed in mice and rats, have been developed over the last decades (Eltern, 2008).

II-Rodent Models

II-I-Homologous Models

In contrast to humans and non-human primates, estrous animals do not develop endometriosis spontaneously since they do not shed their endometrial tissue. However, endometriosis can be induced by transplanting endometrial tissue to ectopic sites (Grummer, 2006). In autologous models of endometriosis, a part of uterus is removed and cut into small pieces which are then reintroduced, mostly by suturing, into the peritoneal cavity. In most studies, endometrium was not separated from myometrium; thus, both compartments were implanted (Cummings and Metcalf, 1995; Vernon and Wilson, 1985). The uterine tissue develops into fluid-filled, ovoid, cystic structures composed of myometrial and endometrial tissue (Vernon and Wilson, 1985). A limited number of experiments separating the endometrium from the myometrium, only inoculating the endometrium to ectopic sites have been performed in rats (Katsuki *et al.*, 1998) and mice (Hirata *et al.*, 2005; Somigliana *et al.*, 1999).

Autotransplantation of uterine tissue to ectopic sites of small laboratory animals has been established not only in rats (Jones, 1984; Vernon and Wilson, 1985) and mice (Cummings and Metcalf, 1995; Rossi *et al.*, 2000; Somigliana *et al.*, 1999) but also in hamsters (Steinleitner *et al.*, 1991) and rabbits (Dunselman *et al.*, 1989; Homm *et al.*, 1989; Rock *et al.*, 1993; Schenken and Asch, 1980). The first autologous endometriosis model was

performed in rabbit in which endometrial fragments from one uterine horn were transplanted on the peritoneum (Schenken and Asch, 1980). After 14 days endometriosis-like lesions developed with the histological features of the disease. Vernon and Wilson (1985) applied this technique on rats, suturing uterine squares on the peritoneum with the same result. In hamsters, two autologous transplantation models were established, one with uterus biopsies on peritoneum (Steinleitner *et al*., 1991) and the other with endometrial fragments placed on the striated skin muscle in a dorsal skin-fold chamber (Laschke *et al*., 2006).

Adapted from the first rat model (Vernon and Wilson, 1985), the first autologous mouse model was described in 1995 by Cummings and Metcalf (1995). In this model, one uterus horn was excised and cut into small fragments which were then sutured in the same animal on the peritoneal cavity. The advantage of this model was the high take rate due to fixed lesions and the ease of recovering them. Also, endometrium alone was used to establish endometriosis by injection into the peritoneal cavity in mice (Somigliana *et al*., 1999). According to this procedure isolated endometrial tissue was minced and reintroduced into the peritoneal cavity of recipient, syngeneic animals. Both donor and recipient mice had been oophorectomized and received estrogen treatment. After 3 weeks all recipient animals revealed evidence of peritoneal endometriosis and neovascularization was observed at the surface of the lesions. However, the 'take-rate', that is the number of lesions recovered after a known number of endometrial fragments have been randomly inoculated, was on average 30% of the inoculated tissue.

For better identification of size and localization of ectopic endometriotic lesions after transplantation, Hirata *et al*. (2005) developed a homologous mouse model using 'green mice' that express green fluorescent protein as donors. The fluorescent endometrial tissue was transplanted into recipient mice. The weight of endometriotic lesions significantly correlated with the measured fluorescence intensity. This fluorescence was significantly higher in the estrogen supplemented mice as compared with control animals, supporting the estrogen dependency of these ectopic endometrial lesions. In 2006, Becker *et al*. generated luciferase-expressing transgenic mice by inserting the human ubiquitin C promoter coupled to the firefly luciferase reporter. Injection of luciferin in these mice resulted in full-body bioluminescence. Then, endometrial tissue from these transgenic mice was surgically implanted into nonluminescent recipients. Then, bioluminescence of lesions was noninvasively imaged by intravenous or intraperitoneal injection of luciferin. Also, transabdominal luminescence compared well with the location of the transgenic endometriotic lesions, and lesion size correlated with the intensity of luminescence.

According to Cummings and Metcalf (1995), the ectopic uterine fragments exhibit the histological characteristics of the human disease, including the formation of multiple, highly vascularized lesions containing endometrial glands, stroma and cysts. Additionally, the cysts in rats and women shared many structural and molecular features including the abnormal production in nearby tissues of many substances leading to signs and symptoms of endometriosis. These substances include cytokines and growth factors (Zhang *et al*., 2008).

Rodent models offer many benefits including the possibility of investigating environmental influences like dioxin on the establishment of lesions (Cummings *et al.*, 1996) or of impacts on fertility (Cummings and Metcalf, 1996). Also, these models are highly suitable for examining immunomodulatory drugs and anti-inflammatory compounds (Eltern, 2008).

II-Heterologous Models

In heterologous mouse models, transplantation or injection of non-mouse material in immunodeficient mice was performed. Human endometrium from women with or without endometriosis or fragments from endometriotic lesions were utilized in these models, whether intraperitoneally or subcutaneously (Grummer, 2006; Zamah *et al.*, 1984). Successful establishment of tissue fragments was achived since mice with a defective immune system lack a graft-versus-host reaction (Eltern, 2008).

The first heterologous mouse models have been conducted with athymic "nude" mice (Han:NMRI nu/nu) (Zamah *et al.*, 1984). Later on, SCID and non-obese diabetic (NOD)-SCID mice were utilized, which display a congenital deficiency in T- and B-lymphocyte function (Awwad *et al.*, 1999). The "take-rate" (the number of fragments established from the number of transplanted ones) was higher in SCID mice compared to "nude" mice. Furthermore, lesions showed a better preserved morphology and expression of steroid receptors (Aoki *et al.*, 1994). However, a detectable natural killer (NK) cell activity in these mouse strains existed. So, an immunodeficient strain additionally deficient in NK-cell activity [RAG-2/γ(c)-knockout] was used for heterologous transplantation (Greenberg and Slayden, 2004).

The fact that human endometrial or endometriotic tissue is utilized in these models is valuable for evaluating the aetiology of the disease and for therapeutic testing of pharmacological approaches. Despite these advantages, investigating the impact of the immune system on lesion establishment is not possible in these models. For this purpose, autologous models in non-immunosuppressed mice are helpful (Grummer, 2006).

The chicken Chorioallantoic Membrane Model

The chicken chorioallantoic membrane (CAM) model was developed originally to study the invasive, metastatic and angiogenic potential of neoplastic cells (Scher *et al*., 1976). Later on, it has been utilized as a model for endometriosis by culturing fragments of human endometrial tissue on the basal layer of the CAM of fertilized chicken eggs (Maas *et al*., 2001). So, it can be considered as an animal model in the broader sense.

Endometrial fragments from the proliferative and secretory phase of the menstrual cycle as well as the menstrual endometrium were able to invade across the epithelium into the mesenchymal layer. Endometriosis-like lesions developed in this layer of the CAM within 3 days (Maas *et al*., 2001; Nap *et al*., 2003). However, the presence of intact glandular structures and stromal components in the cultured endometrium is a requirement for successful growth (Nap *et al*., 2003).

The CAM assay is suitable for the investigation of mechanisms involved in invasion and angiogenesis during the first steps in the establishment of endometriotic lesions. So, it is useful to study inhibitors of factors involved in these processes. However, CAM assay is

hardly applicable for the investigation of immunological or inflammatory responses or to analyze long-term drug effects in the course of the disease (Grummer, 2006).

2.6. Tested Compounds

2.6.1 Caffeine

Caffeine; a constituent in coffee, tea, and soft drinks, is the world's most widely consumed pharmacologically active substance (Scheindlin, 2007). It exerts its action mainly through antagonism of the effects mediated by the adenosine receptor subtypes A_1, A_{2A}, A_{2B} and A_3 (Varani *et al.*, 2006). Caffeine acts most potently at A_{2A} receptors, followed closely by A_1 receptors, then A_{2B} receptors, and as a weak antagonist at human A_3 receptors (Varani *et al.*, 2000).

Caffeine exerts its effects through the blockade of adenosine receptors located on cell membranes of the central nervous system, blood vessels, platelets, neutrophils and other tissues. Its pharmacological activities include increasing vigilance, decreasing psychomotor reaction time, increasing sleep latency and waking time, relaxation of smooth muscles, enhancement of of gastric acid secretion and release of catecholamines, and increasing metabolic activity (Varani *et al.*, 2000). Tolerance to the effects of caffeine develops rapidly. Also, when caffeine is discontinued, withdrawal symptoms occur, mainly including headache and fatigue, and less commonly anxiety, impaired psychomotor performance, nausea or vomiting, or an intense desire for coffee (Scheindlin, 2007).

Biological Activities of Caffeine

I- Central Nervous System Stimulatory Effect

An over-the-counter indication for caffeine is recognized in the monograph "Stimulant Drug Products for Over-the-Counter Human Use". Actually, the only stimulant approved to help restore mental alertness or wakefulness when experiencing fatigue or drowsiness is caffeine (Scheindlin, 2007).

II- Cardiovascular Effects

Evidence exists that caffeine at dietary doses increases blood pressure. It caused systolic and diastolic blood pressure elevations in women (4.5 and 3.3 mm Hg, respectively) and men (4.1 and 3.8 mm Hg, respectively) (Hartley *et al.*, 2004). The effect of chronic caffeine consumption on platelets depends on both dose and duration of the treatment and results in a reduction in platelet aggregability due to upregulation of the adenosine A_{2A} receptors (Varani *et al.*, 2000).

III- Treatment of Apnea in Newborns

A prescription-only use of caffeine is the treatment of apnea in newborns. In premature babies, episodes of apnea tend to occur frequently. Both caffeine and theophylline are equally effective in regulating breathing, but caffeine is easier to administer, has a wider therapeutic index, and produces fewer peripheral effects (Scheindlin, 2007).

IV-Effect of Caffeine on Angiogenesis

Adenosine receptor antagonism caused inhibition of angiogenic activity. In fact, adenosine is a known mediator of angiogenesis in hypoxic tissues (Barcz *et al*., 2000). Furthermore, it has been demonstrated that caffeine can potentiate the anti-angiogenic effect of epigallocatechin gallate; the primary polyphenol in green tea extract, (Sagar *et al*., 2006).

V-Anti-Inflammatory Activity

In analgesic products, caffeine has long been used as an adjuvant. It is believed that it enhances their absorption and potentiates their anti-inflammatory activity (Scheindlin, 2007). A number of *in vitro* and *in vivo* studies demonstrated that caffeine modulates both innate and adaptive immune responses via inhibition of cAMP-phosphodiesterase (Sabisz and Skladanowski, 2008). On the other hand, Horrigan *et al*. (2004) investigated the effect of *in vitro* exposure to caffeine at concentrations relevant to typical caffeine consumption in humans, on lipopolysaccharide (LPS)-stimulated cytokine production in human whole blood. Caffeine had little or no effect on interleukin (IL)-10, IL-1β, or IL-12 production. However, TNF-α production was suppressed and this effect was mediated by the cyclic AMP/protein kinase A pathway *in vitro.*

VI-Reciprocal Roles between Caffeine and Estrogen on Bone

Caffeine decreased bone mineral density, negatively influence calcium absorption and increased the risk of bone fracture in women. Interestingly, caffeine may exert its undesirable effects on bone only in absence or low level of estrogen *in vivo* and estrogen may antagonize these adverse effects (Zhou *et al*., 2009).

VII-Effect of Caffeine on Tumorigenesis

It is well documented that caffeine modulates carcinogenesis at various organ sites including liver, skin, lung, and mammary gland, in animals treated with carcinogens (IARC, 1991). It has been suggested that the induction of cytochrome P450 enzymes in the rat liver may account for its anti-cancer activity (Chung *et al.*, 1998). Caffeine also protected against ultra violet (UV) light-induced skin tumorigenesis (Huang *et al.*, 1997).

2.6.2. Beta-Caryophyllene

Beta-Caryophyllene is a bi-cyclic sesquiterpene with a rare 1,1-dimethylcyclobutane ring fused in a trans configuration to a nine-membered ring containing a 1,5-diene (Figure 7) (Collado *et al.*, 1998). In nature, β-caryophyllene is usually found together with small quantities of its isomers isocaryophyllene and α-humulene (formerly known as α-caryophyllene) or in a mixture with its oxidation product, β-caryophyllene oxide (Gertsch *et al.*, 2008).

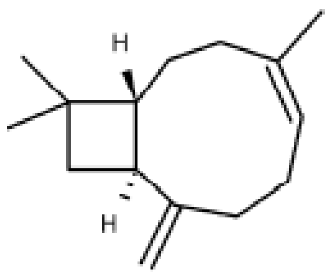


Figure 7. Structure of β-caryophyllene (Collado *et al.*, 1998).

Beta-Caryophyllene is a major volatile compound found in large amounts in the essential oil of *Cannabis sativa* L. (up to 35%) (Hendriks *et al.*, 1975). This widespread natural product is found also in different spice and food plants, such as *Eugenia caryophyllata*, 1992), oregano (*Origanum vulgare* L.), cinnamon (*Cinnamomum* spp.), *Zingiber nimmonii* and black pepper (*Piper nigrum* L.) (Gertsch *et al.*, 2008). Due to its spicy odour, β-caryophyllene is commonly used as a flavouring agent and as fragrance in cosmetics such as creams, lotions, and soaps (Verghese, 1994). Furthermore, it is used as an additive to tobacco products for inclusion in cigarettes (Morris, 2005).

Biological Activities of β-Caryophyllene

I-Anti-Inflammatory Activity of β-Caryophyllene

Beta-caryophyllene exerted anti-inflammatory activity in the rat hindpaw edema model induced by the phlogogen agents, carrageenan or prostaglandin E_1 in rats. Furthermore, it reduced carrageenan-induced inflammation in adrenalectomized animals (Martin *et al.*, 1993). Also, it inhibited LPS-induced proinflammatory cytokine expression in peripheral blood and decreased LPS-stimulated Erk1/2 and JNK1/2 phosphorylation in monocytes (Gertsch *et al.*, 2008). Beta-caryophyllene exerted a potent anti-inflammatory effect in wild type mice but not in cannabinoid type-2 (CB_2) receptor ($Cnr2^{-/-}$) knockout mice. It selectively bound to the CB_2 receptor and acted as a nonpsychoactive full CB_2 receptor agonist *in vivo* (Gertsch *et al.*, 2008).

II-Anti-allergic Activity of β –Caryophyllene

β-Caryophyllene was four times as active as the antiallergic drug tranilast against passive cutaneous anaphylaxis induced in the ears of mice. Also, it was more effective than its structural isomer, α-humulene (Tanaka *et al*., 1996). However, preventive or therapeutic treatment with β-caryophyllene has not significantly reduced eosinophil recruitment to the bronchoalveolar lavage fluid (Rogerio *et al*., 2009).

III-Anti-Cancer Activity of β -Caryophyllene

Beta-Caryophyllene showed significant *in vitro* cytotoxicity against human epithelioid cervix carcinoma (HeLa) cells and human breast carcinoma (BT-20) cells. Moderate cytotoxicity was also exhibited against human melanoma skin cells (HTB-140) and mouse melanoma cells (B-16) (Kubo *et al*., 1996). Furthermore, it exerted cytotoxic activity on colon cancer HCT-116 but in the breast cancer MCF-7 cells it showed less cytotoxicity (Hadri *et al*., 2010). However, β-Caryophyllene was cytotoxic as well against some non-tumor cell lines such as murine macrophage RAW264.7 (Hadri *et al*., 2010).

2.6.3. Beta--Citronellol

Citronellol (Figure 8) is an acyclic monoterpene alcohol prevalent in essential oils of various plants. This monoterpene occurs naturally as two isomeric optical forms. The *R*-(+)-isomer is a common constituent of plant essential oils, especially in the Rutaceae. Conversely, the *S*-(−)-isomer is much less common and it is present in geranium and citronella oils (de Sousa *et al*., 2006).

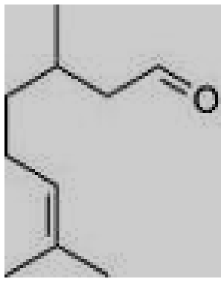


Figure 8. Chemical Structure of β-Citronellol
(www.informahealthcare.com).

Biological Activities of *R*-(+)-Citronellol

I- Anti-Inflammatory Activity

B-citronellol suppressed effectively TNF-α-induced neutrophil adherence at a low concentration (Abe *et al*., 2003).

II-Other Activities

(R)-(+)-citronellal inhibited P-glycoprotein-mediated transport *in vitro*. This transmembrane protein acts as a multidrug resistance factor in tumor cells by transporting certain anticancer agents to the outside of the cells, thereby reducing the intracellular concentration of cytotoxic drugs (Yoshida *et al*., 2005). Also, citronellol possesses an anti-anxiety effect and anti-epileptic effects (de Sousa *et al*., 2006).

2.6.4. Nordihydroguaiaretic Acid (Masoprocol)

Nordihydroguaiaretic acid (NDGA) (Figure 9) is a natural occurring lignan mainly isolated and commercially produced from desert plant, creosote bush (*Larrea divaricata* Cav. Or *Corillea tridentate*). During past 100 years, extensive research has demonstrated that

nordihydroguaiaretic acid is potentially useful in treating diseases related to cancers, diabetes, viral, bacterial infections, and inflammation (Chen, 2009).

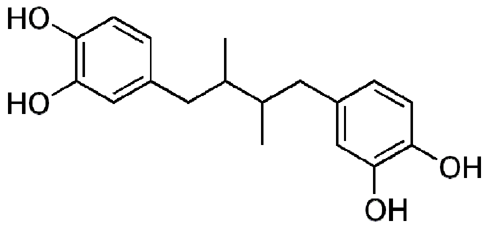


Figure 9. Chemical structure of NDGA

A topical form of NDGA (generic name: masoprocol, trade name: Actinex®) was approved by the Food and Drug Administration for the treatment of actinic keratoses (a neoplastic disease). However, it was later removed from the market following the emergence of skin hypersensitivity (Olsen *et al*., 1991; Kulp-Shorten *et al*., 1993).

Biological Activities of NDGA

I- Antioxidant Activity

Nordihydroguaiaretic acid is a potent *in vitro* scavenger of peroxynitrite, singlet oxygen, hydroxyl radical, superoxide anion and hypochlorous acid and prevented *in vivo* ozone-induced tyrosine nitration in lungs (Floriano-Sánchez *et al*., 2006).

II- Lipoxygenase Inhibitory Activity

Nordihydroguaiaretic acid is well known to be a lipoxygenase inhibitor, as well as an antioxidant (Fujiwara *et al*., 1998). In a rodent model of hypertension, it was able to lower blood pressure and improved the metabolic abnormalities present (Gowri *et al*., 1999).

III- Anti-Cancer

In *in vitro* NDGA has been shown to inhibit cancer cell growth and to have cancer preventive activity in animal models (Hofmanova *et al*., 2002; McDonald *et al*., 2001; Nakadate *et al*., 1989; Park *et al*., 1998). It inhibited several metabolic enzymes known to influence the growth of certain tumors (Maria *et al*., 2006). In addition, NDGA was recently shown to inhibit several receptor tyrosine kinases *in vivo* which are overexpressed in certain cancer cells (Meyer *et al*., 2007; Meyer *et al*., 2008; Youngren *et al*., 2005). Recently, NDGA has been employed clinically in phase I trials with prostate cancer patients (Ryan *et al*., 2008).

III- Other Biological Activities

Masoprocol has been shown to exhibit anti-hyperglycemic activity (Luo *et al*., 1998). Also, it decreases serum triglyceride concentrations in rats (Scribner *et al*., 2000).

2.6.5. Primaquine

Primaquine is the most representative member of the anti-malarial 8-aminoquinoline compounds. It was first synthesized in 1946 and still the only transmission-blocking anti-malarial drug clinically available since that time (Vale *et al*., 2009).

Biological Activities of Primaquine

I-Anti-Malarial Activity

Primaquine is the most effective and the only clinically prescribed drug available against liver stages of *Plasmodium vivax* and *Plasmodium ovale* responsible for malarial relapses (Noela *et al*., 2007). Despite its clinical importance, the utility of this drug has been limited by its side effects such as gastrointestinal disturbances, methaemoglobinaemia and hemolytic anemia, particularly in patients with inborn erythrocytic glucose-6-phosphate dehydrogenase deficiency (Beutler, 1990).

II-Anti-Tumor Activity

Primaquine exhibited significant anti-cancer activity against MCF-7 human breast cancer cell line ($IC_{50} = 6.9\mu M$). It was moderately active against Caco-2 cell line ($IC_{50} = 48\mu M$) but not against HT-29 cell line ($IC_{50} = 69.7\ \mu M$) (Fernandes *et al*., 2009). Moreover, several quinoline-based drugs such as primaquine are often used in combination with anticancer drugs to overcome multi drug resistance in tumors (Kim *et al*., 2005).

III-Anti-Inflammatory Effect

Interest in the use of synthetic antimalarial drugs for the treatment of rheumatoid arthritis and other connective tissue disorders began during the post-World War II era. Primaquine was reported in the medical literature to be an effective drug. However, serious toxic reactions prevented its widespread use (Scherbel, 1983; Steck *et al*., 1952). Palmer and Weatherall (1977) reported the inhibitory activity of primaquine on white blood cell migration. More recently, primaquine was reported to alter the expression of more than one

monocyte receptor. So, it was suggested that it has immunomodulatory action (Goldring and Nemaorani, 1999).

IV-Primaquine is a Receptor Recycling Inhibitor:

Primaquine, with its weak basic properties, strongly inhibits recycling of endocytosed integral membrane proteins and receptors (Van Weert *et al*., 2000). It blocks the secretory pathway at the trans-Golgi network where it interferes with the formation of functional transport vesicles from this acidic compartment (Hiebsch *et al*., 1991). This selective action of primaquine was utilized as a useful tool in studying many membrane receptors that become recycled as in Starke *et al*. (2011).

V-Primaquine Causes Alterations in Gene Expression and Cell Signaling

Primaquine caused transcriptional alterations in the hepatic genes of normal mice. These altered pathways include oxidative stress and FAS pathway, apoptosis, cell cycle, inflammatory response, mitogen-activated protein kinase (MAPK) signaling, matrix metalloproteinases, steroid biosynthesis, transforming growth factor-β (TGF-β) signaling as well as translation factors (Noel *et al*., 2007).

VI-Antioxidant Activity

Primaquine significantly inhibited hepatic superoxide dismutase and catalase which represent the antioxidant system. However, it increased the level of superoxide anion and lipid peroxidation (Srivastava *et al*., 1993).

2.6.6. Sunitinib

Sunitinib (SU-11248, Sutent; Pfizer) is an oral, multitargeted receptor protein-tyrosine kinase (RTK) inhibitor. It selectively and potently inhibits class III and V split kinase domain RTKs, including vascular endothelial growth factor (VEGF) receptors (VEGFR-1, -2, and -3), platelet-derived growth factor (PDGF) receptors (PDGFR-α and -β), stem cell factor receptor (KIT), FMS-like tyrosine kinase-3 receptor (FLT3), the receptor for macrophage colony-stimulating factor (CSF-1R), and glial cell-line–derived neurotrophic factor receptor (rearranged during transfection; RET) (Patyna *et al.,* 2008).

By diminishing signaling through VEGFR1, VEGFR2, and PDGFR-β, sunitinib inhibits angiogenesis. In fact, VEGFR2 is the predominant mediator of VEGF-stimulated endothelial cell migration, proliferation, survival, and enhanced vascular permeability that occur during vasculogenesis and angiogenesis. PDGFR-β, which is found in pericytes that surround capillary endothelial cells, plays an important role in stabilizing the vascular endothelium (Roskoski, 2007). Sunitinib also inhibits receptors such as c-KIT, FLT3 and RET (Figure 10), which are important for the growth of solid tumors and haematologic malignancies (Yang *et al*., 2010).

Biological Activities of Sunitinib

I-Anti-proliferative Activity

Sunitinib has been approved multinationally for the treatment of gastrointestinal stromal tumor after intolerance to or disease progression with imatinib therapy, and for the first-line treatment of advanced renal cell carcinoma (Rock *et al*. 2007). Clinically, sunitinib

antitumor activity was also demonstrated in neuroendocrine and breast cancers (Yang *et al*., 2010).

II-Anti-Angiogenic Activity

VEGF plays an important part in angiogenesis (new blood vessel formation from pre-existing vasculature). The binding and activation of VEGFR on the endothelial cell membrane initiates a signaling cascade that stimulates vessel permeability, endothelial proliferation, migration, and finally blood vessel formation (Kim *et al*., 2008). Angiogenesis plays an essential role in several pathologic conditions, particularly tumorigenesis and metastasis. Growth factors such as VEGF and PDGF or their receptors to which they bind (RTKs) may be overexpressed or mutated in various types of cancer. This leads to disruption of the normal angiogenic balance and creation of a proangiogenic environment (Faivre *et al.,* 2007). For example, renal cell cancers that have metastasized exhibit extensive vascularity. In such neoplasms, sunitinib is approved for their treatment (Roskoski, 2007). In fact, sunitinib leads to stepwise alterations in the endothelial cell layer resulting in blood congestion and necrosis of tumour cells located in the central area of the tumour (Faivre *et al.,* 2007).

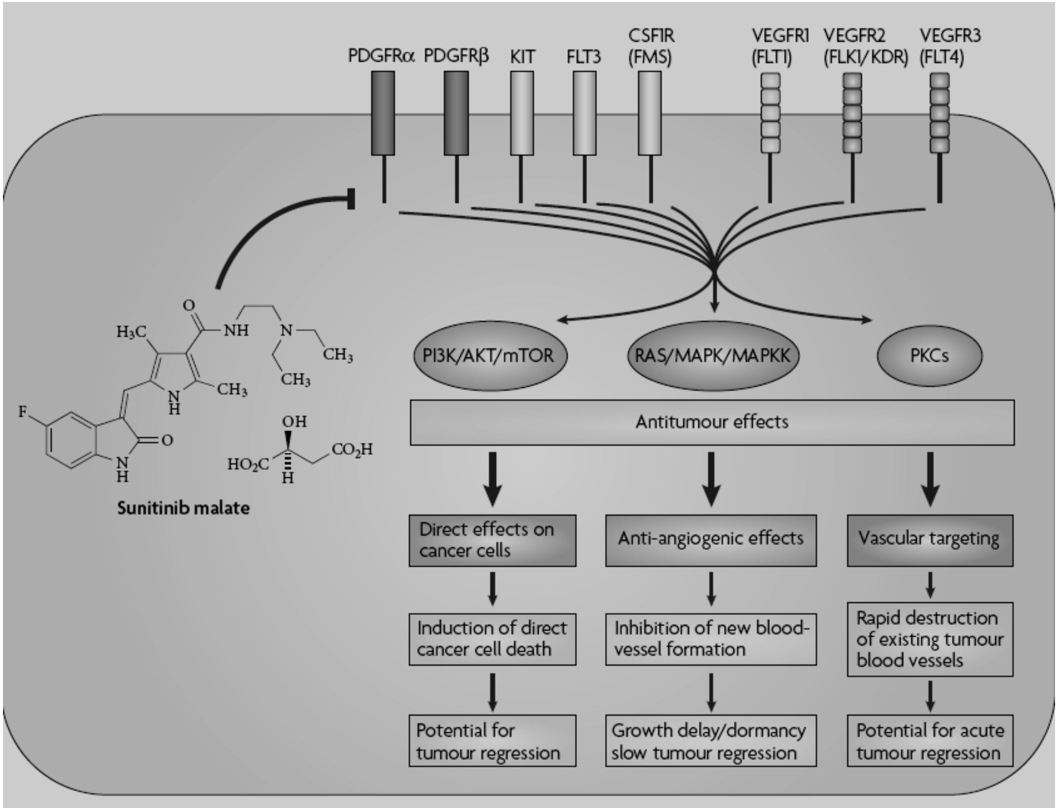


Figure 10. Properties of sunitinib malate. Sunitinib malate interacts selectively with the intracellular ATP-binding sites of tyrosine kinase VEGFR1–3, PDGFRs, KIT, FLT3 and CSF1R. Receptor inhibition has multiple effects on cellular processes including tumour cell survival, endothelial cell growth and migration, vascular permeability, pericyte recruitment and lymphangiogenesis. The final effects may be classified as follows: direct cytotoxic effects on tumour cells by induction of cell death; anti-angiogenic effects leading to growth delay and/or tumour regression by cytostatic inhibition of new blood-vessel formation; vascular disruption by inhibition of existing VEGF/VEGFR-dependant tumour blood vessels leading to central tumour cell necrosis, and cavitation. MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C. (Faivre *et al.,* 2007).

III-Anti-Inflammatory Effect

Recent studies have demonstrated that sunitinib exerts potent immunoregulatory, anti-inflammatory and anti-fibrotic effects (Mao *et al*., 2009). In chronic experimental asthma model, sunitinib significantly inhibited eosinophilic airway inflammation, persistent airway

hyperresponsiveness and airway remodeling. It reduced levels of total serum IgE and BALF Th2 cytokines and also lowered the expression of phosphorylated c-kit protein in remodeled airways (Mao *et al.*, 2009).

2.6.7 Theophylline

Theophylline is a methylxanthine, similar in structure to the common dietary xanthines caffeine and theobromine. Their structural formulas (Figure 11) include a purine ring, as found in the adenine and guanine nucleotides of DNA. The placement of the *N*-methyl groups determines the specific pharmacological profile of each compound (Scheindlin, 2007).

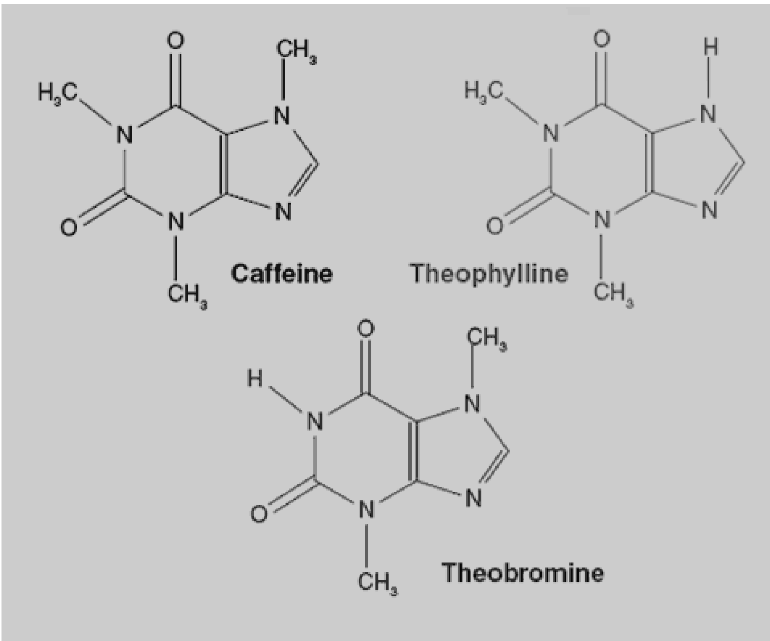


Figure 11. The chemical structures of three common methylxanthines: caffeine, theophylline, and theobromine (Scheindlin, 2007).

Theophylline was used for the treatment of asthma and chronic obstructive pulmonary disease (COPD) for over 70 years (Barnes *et al.*, 2005). Asthma is caused by reduced airway patency secondary to bronchoconstriction and inflammation (Paul *et al.*, 2010). Theophylline was originally used as a bronchodilator. However, the relatively high doses required are associated with frequent side effects, so its use declined (Barnes, 2010). Nowadays, β_2-adrenergic receptor agonists and inhaled corticosteroids become more widely used since the former are far more effective as bronchodilators while the latter have a greater anti-inflammatory effect (Barnes, 2003). According to recent international guidelines, theophylline has been considered a third-line therapy in asthma (Global Initiative for Asthma Guidelines, 2002). This is the case in developed countries, where the cost of the new drugs is no obstacle, but inexpensive theophylline continues to be widely prescribed in other parts of the world (Scheindlin, 2007).

Previously, theophylline was believed to induce bronchodilation by inhibiting phosphodiesterases (PDEs) in pulmonary smooth muscle or via antagonism of the bronchoconstrictor adenosine (Rabe *et al.*, 1995). While theophylline's exact mechanism of action is not defined, a novel molecular mechanism described in 2002 suggested an anti-inflammatory action, rather than bronchodilatory, effects (Ito *et al.*, 2002).

Biological Activities of Theophylline

For a long time, the molecular mechanism of action of theophylline has been a subject of intense debate. Several mechanisms have been proposed, many of which appear to occur only at higher concentrations than are effective clinically (Table 3) (Barnes, 2010).

Table 3. Proposed mechanisms of action of theophylline
(Barnes, 2010).

• Phosphodiesterase inhibition (non-selective)
• Adenosine receptor antagonism (A_1-, A_{2A}-, A_{2B}-receptors)
• Inhibition of nuclear factor-κB (↓ nuclear translocation)
• Inhibition of phosphoinositide 3 kinase-δ
• ↑Histone deacetylase activity (↑ efficacy of corticosteroids)
• ↑ Interleukin-10 secretion
• ↑ Apoptosis of inflammatory cells
• ↓ poly(ADP-ribose)polymerase-1 (inhibits cell death)

I-Phosphodiesterases Inhibitory Action

Theophylline is a weak non-selective inhibitor of phosphodiesterases (PDE), which breaks down cyclic nucleotides in the cell, thereby leading to an increase in intracellular cAMP and cGMP concentrations **(**Barnes, 2010). The molecular mechanism of bronchodilatation is likely explained by PDE inhibition, resulting in an increase in cAMP by inhibition of PDE3 and PDE4 and in cGMP by inhibition of PDE5 (Rabe *et al*., 1995).

II-Adenosine Receptor Antagonism

Theophylline is a potent inhibitor of adenosine receptors at therapeutic concentrations, with antagonism of A_1 and A_2 receptors, although it is less effective against A_3 receptors (Pauwels and Joos, 1995). A novel adenosine monophosphate (AMP) receptor, $P2Y_{15}$, has been identified that is potently inhibited by theophylline (Inbe *et al*., 2004), although the function of this receptor is not yet established (Barnes, 2010).

III-Effects on Gene Transcription

Theophylline prevents the translocation of the proinflammatory transcription factor NF-κB into the nucleus, thus potentially reducing the expression of inflammatory genes (Tomita *et al*., 1999). In human lung mast cells, theophylline, at therapeutic concentrations, inhibited NF-κB activation (Sagara *et al.*, 2001). Inhibition of NF-κB appears to be due to a protective effect against the degradation of the inhibitory protein I-κBα (Ichiyama *et al*., 2001).

IV- Activation of Histone Deacetylases

Expression of inflammatory genes is regulated by the balance between histone acetylation and deacetylation (Barnes *et al*., 2005). In asthma multiple inflammatory genes are activated through proinflammatory transcription factors, such as NF-κB, leading to histone acetylation and increased transcription. This process is reversed by the recruitment of histone deacetylases (HDAC) to the activated inflammatory gene promoter site within the nucleus (Barnes *et al*., 2005). Low-dose theophylline induces activation of HDAC activity both *in vitro* and *in vivo* (Ito *et al*., 2002). This results in suppression of inflammatory genes and proteins, such as granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin-8 (IL-8) that have been switched on by proinflammatory transcription factors, such as NF-κB (Barnes, 2010).

Minimizing the dose of steroid is a goal in chronic asthma and COPD therapy to prevent complications of corticosteroid administration. Clinical trials have explored the beneficial effect of combinations of theophylline with lower doses of corticosteroids as a steroid-

sparing strategy (Paul *et al*., 2010). Watanabe *et al*., (2008) suggested that theophylline itself has anti-inflammatory activity and the glucocorticoid–glucocorticoid receptor system is involved in its anti-inflammatory activity. Theophylline directly activates histone deacetylases (HDACs) which deacetylate core histones that have been acetylated. This results in suppression of inflammatory genes and proteins. Corticosteroids also activate HDACs, but through a different mechanism resulting in the recruitment of HDACs to the activated transcriptional complex *via* activation of the glucocorticoid receptor (Barnes, 2010).

V- Anti-Inflammatory and Immunomodulatory Effects

Theophylline suppressed *in vitro* the generation of reactive oxygen radicals from alveolar macrophages (Dent *et al*., 1994), eosinophils (Hatzelmann *et al*., 1995) and neutrophils (Nielson *et al*., 1990). It inhibited eosinophil degranulation (Hatzelmann *et al*., 1995) and the release of granulocyte-macrophage-colony-stimulating-factor (GM-CSF) from eosinophils (Shute *et al*., 1995). In addition, theophylline inhibited the release of mediators, including platelet-activating factor, Leukotriene C4 (LTC4), cationic proteins, and superoxide anion, from eosinophils, granulocytes, and alveolar macrophages *in vitro* (Calhoun *et al*., 1991; Shute *et al*., 1998; Tenor, *et al*., 1996). Also, theophylline suppressed the release of tumor necrosis factor-α (TNF-α) from blood monocyte and alveolar macrophages (Spatafora *et al*., 1994). Furthermore, it inhibited T-lymphocyte chemotaxis (Hidi-Ng *et al*., 1995) and histamine release from mast cells (Orange *et al*., 1971).

Theophylline had several actions on T-lymphocyte function, suggesting that it might have an immunomodulatory effect. Actually, it stimulated suppressor (CD8+) T-lymphocytes, which may be relevant to the control of chronic airway inflammation (Fink *et al*., 1987; Shohat *et al*., 1983). However, theophylline, suppressed human natural killer activity *in vitro* but not *in vivo* when given in therapeutic doses (Marshall *et al*., 1989).

Yao *et al*., (2005b) analyzed the gene expression profiles of macrophages in response to theophylline. A panel of inflammation related genes was identified, as well as genes associated with angiogenesis, cell adhesion, cell motility, signal transduction, and cell proliferation that are dose-dependently down- or up-regulated by theophylline. So, all the above mentioned studies suggest an anti-inflammatory role of theophylline.

VI-Theophylline Increases Apoptosis in Inflammatory Cells

Prolonged survival of granulocytes due to a reduction in apoptosis may be important in perpetuating chronic inflammation (Barnes, 2003). Theophylline promoted apoptosis in neutrophils *in vitro* (Yasui *et al*., 1997). Also, it inhibited IL-5–dependent eosinophil survival by means of apoptosis in a dose dependent manner (Ohta and Yamashita, 1999). Theophylline also induced apoptosis of T lymphocytes (Ohta and Yamashita, 1999).

VII-Anti-Angiogenic Activity

Adenosine is a known mediator of angiogenesis in hypoxic tissues. Adenosine receptor antagonism caused inhibition of angiogenic activity of human ovarian cancer cells. Using synthetic adenosine receptor antagonists: 8-phenyl-theophylline and 8-cyclopentyl-1,3

dipropylxantine, Barcz *et al*. (2000) established that antiangiogenic properties of theobromine are dependent on its interaction with A_2 adenosine receptor. The structurally related methylxanthine, theophylline is a well known potent inhibitor of A_2 receptors (Pauwels and Joos, 1995). So, it is expected to exhibit such activity. In fact, theophylline and theobromine significantly decreased the angiogenic activity of mononuclear cells obtained from diabetic patients with proliferative retinopathy. Further conformation came from the analysis of gene expression profiles of macrophages in response to theophylline revealed the presence of genes associated with angiogenesis that are dose-dependently regulated by theophylline (Yao *et al*., 2005b).

2.6.8. Thymoquinone

Thymoquinone is the bioactive constituent of the volatile oil of black seed (*Nigella sativa*, Ranunculaceae family) (Abdel-Fattah *et al*., 2000). It is the most abundant component of black seed oil (Sethi *et al*., 2008).

Biological Activities of Thymoquinone

I-Antioxidant Effect

Thymoquinone demonstrated respectable radical scavenging property (Burits and Bucar, 2000). Studies showed that pretreatment with thymoquinone protected organs against oxidative damage induced by a variety of free radical generating agents including carbon tetrachloride evoked hepatotoxicity (Nagi *et al.*, 1999), doxorubicin-induced cardiotoxicity (Nagi and Mansour, 2000) and nephropathy produced by cisplatin (Badary *et al.*, 1997).

II-Antiinflammatory Effect

In animal models, thymoquinone suppressed acetic acid–induced colitis in rats (Mahgoub, 2003), inhibited TNF-α production in murine septic peritonitis (Haq *et al.*, 1999), and reduced carrageenan-induced paw edema in rats (Hajhashemi *et al.*, 2004). Also, it inhibited COX-2 expression and prostaglandin production in a mouse model of allergic airway inflammation (El Mezayen *et al.*, 2006). Furthermore, thymoquinone down-regulated the expression of inducible nitric oxide synthase (El-Mahmoudy *et al.*, 2002).

Thymoquinone has been shown to be a potent inhibitor of eicosanoid generation, namely thromboxane B2 and leucotrienes B4, by inhibiting both cyclooxygenase and lipooxygenase, respectively (Houghton *et al.*, 1995). It has been suggested that thymoquinone action may be mediated in part through the suppression of the NF-κB activation pathway (Sethi *et al.*, 2008).

III-Chemopreventive Effect

Thymoquinone was found to inhibit proliferation in numerous cell lines *in vivo* and *in vitro.* The inhibitory effects of thymoquinone against 20-methylcholanthrene (MC)-induced fibrosarcoma (Badary and Gamal, 2001) and against benzo(a)pyrene-induced forestomach carcinogenesis (Badary *et al.*, 1999) were reported in mice. Thymoquinone supplementation induced quinone reductase and glutathione transferase in mice liver. This may explain the role of thymoquinone in protection against chemical carcinogenesis and toxicity (Nagi and Almakki, 2009).

Thymoquinone has been shown to suppress the proliferation of various tumor cells including colorectal carcinoma (Gali-Muhtasib *et al*., 2004), breast adenocarcinoma, osteosarcoma (Roepke *et al*., 2007), ovarian carcinoma, myeloblastic leukemia (El-Mahdy *et al*., 2005), and pancreatic carcinoma (Tan *et al*., 2006), although it is minimally toxic to normal cells (Worthen *et al*., 1998). Furthermore, it has been demonstrated that the growth of prostate cancer cells is highly sensitive to the inhibitory effect of thymoquinone, and that this inhibitory action is extremely selective, showing very little effect on the growth of noncancerous prostate epithelial cells in culture, and preventing the growth of human prostate tumors in nude mice (Kaseb *et al*., 2007).

IV- Other Activities

Thymoquinone inhibited angiogenesis *in vitro* and *in vivo* (Yi *et al*., 2008). Also, it produced antinociceptive effects (Abdel-Fattah *et al*., 2000). Black cumin oil was found to decrease plasma concentrations of cholesterol and triglycerides due to the high content of thymoquinone (Ali and Blunden, 2003). Furthermore, thymoquinone supplementation attenuates hypertension and renal damage in nitric oxide deficient hypertensive rats (Khattab and Nagi, 2007). *Nigella sativa* thymoquinone-rich fraction greatly improved plasma antioxidant capacity and expression of antioxidant genes in hypercholesterolemic rats (Ismail *et al*., 2010).

2.6.9. Vitamin D

There are two sources of vitamin D in humans: Dietary sources, including ergocalciferol (D_2) and cholecalciferol (D_3), and endogenous production by the skin in response to

sunlight (D_3 only) (Stubbs *et al*., 2010). Solar ultraviolet B photons are absorbed by 7-dehydrocholesterol in the skin, leading to its transformation to previtamin D3 (Holick, 2004). To become active, vitamin D (D_2 and D_3) must undergo hydroxylation in the liver to form 25-hydroxyvitamin D, a prehormone that is further hydroxylated *via* the 1α-hydroxylase enzyme in the kidney to generate 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$), the active form of this hormone. A separate enzyme, 24-hydroxylase is a deactivating enzyme that forms an inactive metabolite that is excreted in the bile (Stubbs *et al*., 2010).

Vitamin D level is determined by exposure to the solar UV-B irradiation and to a much lesser degree to oral intake of vitamin D (Pearce and Cheetham, 2010). In fact, vitamin D inadequacy is a global problem (Holick, 2007; Mithal *et al*., 2009). Approximately 36% of otherwise healthy young adults and up to 57% of general medicine inpatients in the United States suffer from vitamin D inadequacy. These figures are even higher in Europe (Zhang and Naughton, 2010). Interestingly, the proportion of subjects with vitamin D levels below 25nmol/L was 62.3% in a study conducted in Jordan (Mishal, 2001).

Biological Activities of Vitamin D

Vitamin D is a major regulator of mineral ion homeostasis by facilitating calcium and phosphate uptake in the gut and by modulating bone cell development and action (Kumar 1980; Jones *et al.* 1998). So, vitamin D is usually used for the prevention of rickets in children (Zhang and Naughton, 2010).

It was noticed that children with nutritional rickets were more likely to experience infections of the respiratory system. So, a link between vitamin D deficiency and susceptibility to infection has been suggested for more than a century. In the 1930s, vitamin D_3, isolated from cod liver oil, was used to treat tuberculosis. It was used then as anti-infective chemotherapy in the 1950s. More recently, epidemiologic studies have demonstrated strong associations between seasonal variations in vitamin D levels and the incidence of various infectious diseases, including respiratory infection and influenza (Yamshchikov *et al*., 2009).

The most potent metabolite of vitamin D, ($1,25(OH)_2D_3$), has also been demonstrated to affect a wide range of functions not immediately linked to calcium homeostasis (Vigano *et al*., 2006b ; Zehnder *et al.* 2001). In fact, $1,25(OH)_2D_3$ is involved in regulation of cell differentiation, proliferation, cellular growth, and hormone secretion (Holick, 2004). Also, vitamin D deficiency has also been linked with the pathogenesis and/or progression of several disorders, including cancer, hypertension, multiple sclerosis and diabetes, although the evidence for the associations of vitamin D with these conditions is generally weaker than it is for bone-related disease (Zhang and Naughton, 2010). Experimentally, it was proven that $1,25(OH)_2D_3$ reduces the incidence of diabetes, attenuates murine lupus, has anti-proliferative effect in psoriasis and antineoplastic effect in prostate cancer (Lemire, 2000).

Vitamin D as an Immunomodulator

The discovery that vitamin D receptor (VDR) and 1α-hydroxylase, the enzyme necessary for conversion of vitamin D into its active form, are present in cells of the immune system has revolutionized the field of vitamin D immunology (Yamshchikov *et al.*, 2009). In fact, VDR was present in most cell types of the immune system, in particular in antigen presenting cells such as macrophages and dendritic cells, as well as in both CD4+ and CD8+ T cells. Furthermore, it was found that mice incapable of synthesizing $1,25(OH)_2D_3$ because of the targeted ablation of the 25-hydroxyvitamin D 1α-hydroxylase enzyme show a significant reduction in CD4+ and CD8+ peripheral T cells (Mathieu and Adorini, 2002). Actually, this has led to the investigation of the potential for $1,25(OH)_2D_3$ as an immunomodulatory agent.

Vitamin D regulates the expression of specific endogenous antimicrobial peptides in immune cells; this action leads to a potential role for vitamin D in modulating the immune response to various infectious diseases (Yamshchikov *et al.*, 2009). Induction of monocyte differentiation and the inhibition of antigen presentation by antigen presenting cells have been demonstrated *in vitro*, inhibition of T-cell proliferation and cytokine production, and inhibition of secretion of antibodies by B-cells. At a cellular and molecular level, the hormone preferentially targets helper T cell activity (Th1) by inhibiting the secretion of both IL-2 and interferon- γ (IFN-γ) by Th1 and by suppressing the secretion pro-Th1 cytokine IL-12 by antigen-presenting cells. The active metabolite further inhibits class II antigen expression and enhances suppressor cell activity (Lemire, 2000).

Under experimental conditions, 1,25-$(OH)_2D_3$ is strongly immunosuppressive and improves various T-helper-1 triggered diseases including autoimmune encephalomyelitis and autoimmune diabetes in mice and psoriasis in humans. Moreover, it strongly inhibits the function of natural killer cells and promotes T-helper-2 differentiation leading to a T-helper-2 phenotype with augmented production of interleukin (IL)-4, IL-5 and IL-10, and reduced synthesis of IFN-γ (Lehman, 2005). These *in vitro* effects are reflected *in vivo* by a potential to prevent autoimmune diseases in different experimental models (e.g. autoimmune diabetes in non-obese diabetic mice, experimental autoimmune encephalitis, collagen-induced arthritis) and to prolong graft survival (e.g. pancreatic islets, heart, liver, skin grafts (Casteels *et al*. 1995; Mathieu and Adorini, 2002).

A major problem with the *in vivo* use of 1,25-$(OH)_2D_3$ is the fact that the doses needed to see non-classical effects, such as the immune effects, are high and result in severe hypercalcaemia and accelerated bone remodeling. Due to the flexible structure of 1,25-$(OH)_2D_3$, however, chemists have succeeded in synthesizing analogues of the molecule, some of which share the mother molecule's effects on the immune system, but not its effects on calcium or bone metabolism (Bouillon *et al*. 1995; van Etten *et al*., 2002; Verstuyf *et al*. 2000).

Vitamin D and Endometriosis

I-Level of Vitamin D and vitamin D-binding protein in Endometriosis

In 1990, Hartwell *et al*. reported higher serum levels of 1,25-$(OH)_2D_3$ and similar levels of 25-hydroxyvitamin D in women with endometriosis when compared to controls. This was

the first observation for a possible link between endometriosis and the vitamin D system. However, it remained the only observation for up to 15 years. Recently, it has been demonstrated that human endometrium can be included among those sites capable of extra-renal synthesis of active vitamin D (Vigano *et al*., 2006b). One alpha-hydroxylase; the enzyme that catalyzes the synthesis of 1,25-$(OH)_2D_3$, was expressed in both eutopic and ectopic endometrium. In fact, there was a significantly higher 1-α-hydroxylase expression in the endometrium of patients with endometriosis compared to healthy controls. So, it was suggested that vitamin D might influence the local activity of immune cells and cytokines thought to play important pathogenic roles in the development and maintenance of endometriosis (Agic *et al*., 2007). Further confirmation came from the measurement of 1,25-$(OH)_2D_3$ levels in the supernatant of endometrial cells treated with 25-hydroxyvitamin D. This indicates that the endometrium represents a site of local conversion from the precursor to the active form (Vigano *et al*., 2006b).

Somigliana *et al*. (2007) studied serum levels of 25-hydroxyvitamin D, 1,25-$(OH)_2D_3$, and Ca^{2+} in women with endometriosis. Higher levels of 25-hydroxyvitamin D_3 in the serum of women with endometriosis were observed. A biologic gradient indicating more striking differences in patients with advanced stages was also noted. So it was concluded that endometriosis is associated with higher serum levels of vitamin D. However, Agic *et al*. (2007) found no differences in 25-hydroxyvitamin D_3 levels between the serum of patients with endometriosis and healthy controls.

Similarly, studies evaluating vitamin D-binding protein level in endometriosis gave variable results. According to Ferrero *et al*. (2005), vitamin D binding protein in the

peritoneal fluid but not in plasma decreased in women with untreated endometriosis. On the other hand, Borkowski *et al*. (2008) reported that serum and peritoneal vitamin D binding protein concentrations were not affected in endometriosis patients. Proteomic analysis of serum of women undergoing surgery revealed that the abundance of vitamin D-binding protein was higher in all endometriosis pools by a factor of approximately 3 compared with the control pool (Faserl *et al*., 2011).

II-Vitamin D Receptor and Endometriosis

The biological effects of 1,25-$(OH)_2D_3$ are mediated by the Vitamin D Receptor (VDR), a member of the superfamily of nuclear hormone receptors. Ligand binding induces conformational changes in the VDR, which promote heterodimerization with the retinoid X receptor and recruitment of several nuclear receptor coactivator proteins, including steroid receptor coactivator family members and a multimember coactivator complex, D receptor interacting proteins. These coactivators induce chromatin remodeling through intrinsic histone-modifying activities and direct recruitment of key transcription initiation components at regulated promoters. So, VDR functions as a ligand activated transcription factor that binds to specific DNA sequence elements (vitamin D responsive element) in vitamin D responsive genes and ultimately influences the rate of RNA polymerase II mediated transcription (Mathieu and Adorini, 2002).

Immunohistochemistry demonstrated strong staining of the Vitamin D Receptor in endometriosis with the most intense staining in epithelial cells (Agic *et al*., 2007). Furthermore, since various cell types involved in immunologic reactions (monocytes,

Langerhans cells, T and B lymphocytes) not only express vitamin D receptor but also possess 1α-hydroxylase activity (Van Etten *et al*., 2003; Lehman, 2005), it was suggested that vitamin D might influence the local activity of immune cells and cytokines thought to play important pathogenic roles in the development and maintenance of endometriosis (Agic *et al*., 2007).

III-Vitamin D is often Given to Endometriosis Patients as a Supplement to Prevent Osteoporosis

Endometriosis is treated by inducing a hypoestrogenic state. Endometriosis itself is not a risk factor for reduced bone density. However, its medical treatment, especially with GnRH agonists, is associated with reduced bone density (DiVasta *et al*. 2007; Tudor-Locke and McColl, 2000). It was reported that GnRH agonist as drug therapy reduces bone mass by about 2–5% when used for 6 months (Seko *et al*., 2004). Since GnRH agonists are used after peak bone mass is reached, this decrease in bone mass during this period can serve as a significant risk factor for postmenopausal osteoporosis (Seko *et al*., 2004). Also, drugs that act as aromatase inhibitors decrease local estrogen synthesis in endometriosis. So, it was suggested that they also would reduce ovarian estrogen production and may therefore require estrogen add-back therapy to protect bones (Chwalisz *et al*., 2002). Therefore, vitamin D is given sometimes in endometrioisis as a supplement to prevent osteoporosis (Crosignani *et al*., 2006).

Materials and Methods

3.1. Animals

All animal procedures were conducted in accordance with Jordanian regulations for animal experimentation and care and approved by the committee of institutional animal care and use. Virgin female rats (*Rattus norvegicus*) weighing 180-250 gm were obtained from The University of Jordan animal house. They were allowed to acclimatize to the laboratory conditions for at least two weeks before use. Food and water were provided *ad libitum*. The temperature was maintained between 18 ºC and 25 ºC.

3.2. Chemicals

The following chemicals were purchased from Sigma-Aldrich, USA: Caryophyllene (≥98.5%), primaquine (98%), cholecalciferol (vitamin D_3), nordihydroguaiaretic acid ≥97%, thymoquinone, aprotinin from bovine lung (lyophilized), thrombin from bovine plasma (lyophilized), 6-aminocaproic acid, gentamicin solution (suitable for cell culture), dimethyl sulfoxide for molecular biology ≥ 99.9%, suramin sodium salt ≥ 95%, phosphate buffer saline and L-glutamine. Theophylline was purchased from Siegfried Ltd, Zofingen, Switzerland, Sunitinib (malate salt) from LC laboratories, USA, chloralhydrate from Medical Export Co Ltd, UK and caffeine from Merck, Germany. Amphotericin B was purchased from PAA-The Cell Culture Company, USA. Fibrinogen from bovine plasma from calbiochem, USA. Fetal Bovine Serum (EU Approved Origin) Gibco, USA. Medium 199 powder (10x1L) - with Earle's salt & L-glutamine, without sodium bicarbonate- Gibco, USA-Bio Diagnostics Sdn. Bhd. Tissue Culture multi-dish 48 wells was purchased from Nunclon surface Corning, USA-FC Bios Sdn Bhd.

3.3. Vaginal Smear

Vaginal smears were performed as in Marcondes *et al*. (2002). Briefly, smears were taken daily between 10:00 a.m. and 1:00 p.m. Vaginal secretion was collected with a plastic pipette filled with about 100 µL of 0.85% NaCl by inserting the tip into the rat vagina. Vaginal fluid was placed on glass slide. Unstained smear was observed under a light microscope, with 10 x objective lens. Three types of cells could be recognized (Figure 12): Round and nucleated ones are epithelial cells; irregular ones without nucleus are the cornified cells; and the little round ones are the leukocytes. The proportion of the 3 cell types was used for the determination of the estrous cycle phases. Only those females which exhibited a regular 4-5 day estrous cycle underwent surgery.

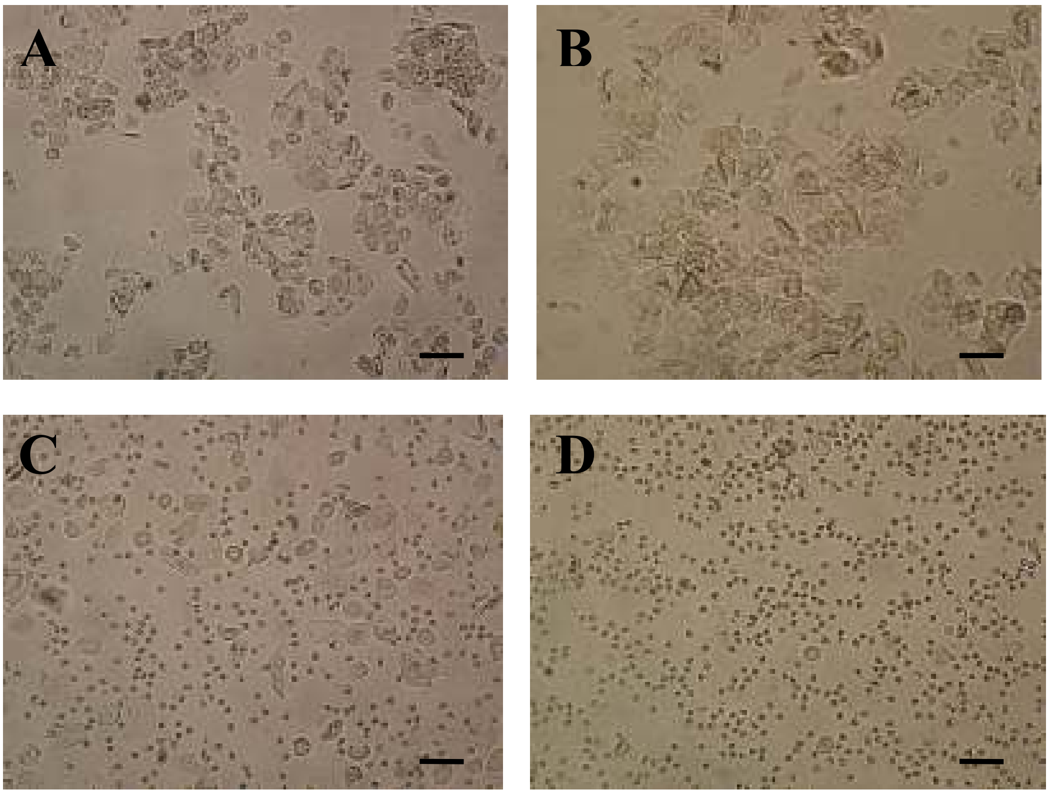


Figure 12. Stages of the estrous cycle. (A) Proestrus (B) Estrus (C) Metestrus (D) Diestrus. Scale bar =50µm.

3.4. First Surgery: Induction of Endometriosis

For the induction of endometriosis, the technique of Vernon and Wilson (1985) was followed with slight modifications. Briefly, a 3 cm ventral incision was performed aseptically under chloralhydrate anesthesia (300 mg/kg given intraperitoneally). Resection of the right uterine horn was achieved after ligation at the utero-tubal junction and the utero-cervical junction. The excised horn was immersed in sterile 0.85% NaCl and the endometrium was exposed by lengthwise incision (Figure13a). Two squares of 4 x 4 mm of open uterus were prepared. Each square was sutured to abdominal muscles with the endometrium facing the peritoneal cavity. The fragment was always fixed over a large blood vessel (Figure 13b). A single stitch using 4-0 nylon suture (Hospital & Homecare Imp. & Exp. Co., Ltd., China) was applied at two opposite sides of the "square". In all experiments, about 2 ml of sterile 0.85% NaCl were added to the abdominal cavity to prevent dehydration and to minimize adhesions. The abdominal wall was closed in two layers using 4/0 nylon suture.

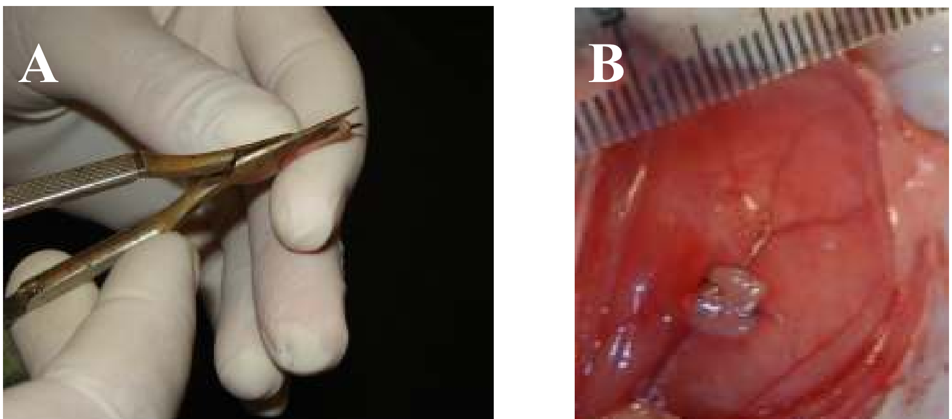


Figure 13. Transplantation of an endometrial implant
(A) A segment of uterus is cut longitudinally using a microscissor and fixed over a blood vessel using a surgical suture (B)

3.5. Second Surgery

Second surgery was performed 28 days after the first one. Briefly, under aseptic conditions a 3 cm ventral incision was made for each rat under chloralhydrate anaesthesia. The the two major diameters were measured by a caliper (accuracy = 0.05 mm). Then, the cross sectional area was calculated using the formula for an ellipsoid [length x width x (π/4)] (Eltern, 2008). Then the abdominal wall was closed in two layers using 4/0 nylon suture and 2 ml of sterile 0.85% NaCl were added to the abdominal cavity.

After the second surgery, animals were randomly divided into different groups to receive one of the nine compounds or vehicle for 21 days. Bilateral oophorectomy was performed to a group of rats after cyst development. This group served as a positive control. At least ten animals were used per group. However, any animal that developed any complications after the first and/or second surgery was excluded (e.g wound infection). Treatment started immediately after the second surgery by single daily intraperitoneal injection of the tested compound. Each treatment group had its control run at the same time and received the same vehicle. Detailed information about dosage is outlined in table 4.

Table 4. Protocol of treatment

Group	Treatment
Group 1	caffeine 10 mg/kg in 0.85% NaCl
Group 2	caryophyllene 10 mg/kg in 2.5% tween 20
Group 3	caryophyllene 30 mg/kg in 2.5% tween 20
Group 4	citronellol 10 mg/kg in 2.5% tween 20
Group 5	NDGA* (masoprocol) 10 mg/kg in dimethylsulfoxide (DMSO)
Group 6	primaquine 10 mg/kg in 0.85% NaCl
Group 7	sunitinib 10 mg/kg in 0.85% NaCl
Group 8	theophylline[§] 10 mg/kg in 0.85% NaCl
Group 9	theophylline 15 mg/kg in 0.85% NaCl
Group 10	thymoquinone 1 mg/kg in DMSO
Group 11	thymoquinone 2 mg/kg in DMSO
Group 12	cholecalciferol (vitamin D) 42 µg/kg in DMSO
Group 13	0.85% NaCl (negative control)
Group 14	DMSO (negative control)
Group 15	2.5% tween 20 (negative control)

* NDGA was prepared immediately before use to exclude the possibility of rapid oxidation.
[§]Theophylline was briefly pre-warmed before use to ensure that it was dissolved completely.

3.6. Light Microscopic Studies

Endometrial explants (cysts) were stored in 10% formalin solution for histological evaluation. Four-five micometer-thick sections were prepared and stained with haematoxylin and eosin as in Appendix I. In addition, sections were stained with Masson trichrome stain (Appendix II) to evaluate the degree of fibrosis. For the evaluation of pathological scores for stromal tissue, a scoring system modified from that used by Altintas *et al*. (2008) was followed (Table 5).

Table 5. Grading system for stromal tissue of endometriotic implants

Pathological Score	% of a hpf containing stroma tissue*
0	Absent
1	<25
2	25-50
3	> 50

hpf : high power field.
* 5 hpfs were counted and the average of them was calculated.

Mast cells stain metachromatically with toluidine blue because their granules contain heparin and sulfated glycosaminoglycan (Sugamata *et al*., 2005). The 4-5 µm sections were stained for 10 min with toluidine blue (Gurr, BDH Chemicals Ltd, UK) as in Appendix III. The pH was adjusted to 2.4 with HCl. Sections were then examined by light microscope.

3.7. Electron Microscopic Studies

For electron microscopy, specimens were fixed in Karnovsky fixative (Karnovsky, 1965) as in Appendix IV, embedded in Spurr's medium, cut into 70-90 nm thick silver-gold sections with ultracut E Reichert Tung ultramicrotome and stained with aqueous uranyl acetate (30 minutes) and Renyold’s lead citrate (15 minutes) and viewed using Zeiss electron microscope 10 CR/ Yarmouk University, Jordan.

3.8. DeadEnd™ Colorimetric TUNEL Assay

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay for the detection of apoptosis was done using the DeadEnd™ Colorimetric TUNEL System from Promega, USA according to the manufacturer's instructions.

Assay principle

The DeadEnd™ Colorimetric TUNEL System end-labels the fragmented DNA of apoptotic cells using a modified TUNEL assay. Biotinylated nucleotide is incorporated at the 3´-OH DNA ends using the terminal deoxynucleotidyl transferase, recombinant, (rTdT) enzyme. Horseradish peroxidase-labeled streptavidin (Streptavidin HRP) is then bound to these biotinylated nucleotides, which are detected using the peroxidase substrate, hydrogen peroxide, and the stable chromogen, diaminobenzidine (DAB). Using this procedure, apoptotic nuclei are stained dark brown. Composition of buffers used in TUNEL assay is given in Appendix V.

Procedure

1. Four-five paraffin-embedded sections were attached to Menzel-Glaser SuperFrost Plus slides, Germany. Then, wax was melted in an oven at 60 ºC for 45 minutes.
2. Sections were deparaffinized by immersing the slides in fresh xylene in a Coplin jar for 5 minutes at room temperature (two changes).
3. The slides were immersed in 100% ethanol for 5 minutes at room temperature in a Coplin jar (three changes).
4. Samples were rehydrated by sequentially immersing the slides through graded ethanol washes (95%, 85%, 70% and 50%) for 3 minutes each at room temperature.

5. Slides were washed by immersing the slides in 0.85% NaCl for 5 minutes at room temperature then by immersing them in PBS for 5 minutes at room temperature.

6. Tissue sections were fixed by immersing the slides in 10% buffered formalin in PBS for 15 minutes at room temperature.

7. Slides were immersed in PBS for 5 minutes at room temperature (two changes).

8. The liquid was removed from the tissue and the the slides were placed on a flat surface.

9. A 20µg/ml proteinase K solution was prepared from the 10mg/ml proteinase K stock solution by diluting 1:500 in PBS. One hundred microliters of the 20µg/ml proteinase K solution were added to each slide to cover the tissue section and slides were incubated for 10–30 minutes at room temperature. Proteinase K helps permeabilize tissues, but prolonged incubation may cause sections to come off the slide. So, the length of incubation was optimized to 16 minutes.

9. The slides were washed by immersing in PBS for 5 minutes at room temperature in a Coplin jar.

10. The tissue sections were refixed after washing by immersing the slides in 10% buffered formalin in PBS for 5 minutes at room temperature.

11. The slides were washed by immersing in PBS for 5 minutes at room temperature (two changes).

12. Excess liquid was removed by tapping the slides. The cells were covered with 100µl of equilibration buffer for 5–10 minutes.

13. While the sections are equilibrating, the biotinylated nucleotide mixture was thawed on ice and sufficient rTdT reaction mixture was prepared for all experimental and control reactions and kept on ice. rTdT reaction mixture was prepared by adding 98µl equilibration buffer, 1µl biotinylated nucleotide mixture and 1µl rTdT Enzyme per reaction.

14. Blot around the equilibrated areas with tissue paper and add 100µl of rTdT reaction mix to the sections on a slide. The sections were not allowed to dry.

15. The sections were covered with plastic coverslips to ensure even distribution of the reagent and incubated at 37°C for 60 minutes inside a humidified chamber to allow the end-labeling reaction to occur.

16. The 20X SSC was diluted 1:10 with deionized water. The plastic coverslips were removed and the reactions were terminated by immersing the slides in 2X SSC in a Coplin jar for 15 minutes at room temperature.

17. The slides were washed by immersing in fresh PBS for 5 minutes at room temperature. This step was repeated twice to remove unincorporated biotinylated nucleotides.

18. The endogenous peroxidases were blocked by immersing the slides in 0.3% hydrogen peroxide in PBS for 3–5 minutes at room temperature.

19. The slides were washed by immersing in PBS for 5 minutes at room temperature (three changes).

20. The streptavidin HRP solution was diluted 1:500 in PBS added to each slide (100µl each) and incubated for 30 minutes at room temperature.

21. The slides were washed by immersing in PBS for 5 minutes at room temperature (three changes).

22. The DAB components were combined just prior to use. Fifty microliters of the DAB substrate 20X buffer were added to 950µl deionized water. Then, 50µl of the DAB 20X chromogen and 50µl of hydrogen peroxide 20X were added. One hundred microliters of DAB solution were added to each slide and develop until there is a light brown background (approximately 10 minutes). The background was not allowed to become too dark. The DAB solution was kept away from light and used within 30 minutes.

23. The slides were rinsed several times in deionized water.

24. Sections were dehydrated using graded concentrations of ethanol and mounted in distrene-plasticizer-xylene (DPX).

3.9. Antiangiogenic Effect of Beta Caryophyllene

The assay was performed according to standard protocol of Brown *et al*. (1996). Briefly, male rats (250 gm) were humanely sacrificed by cervical dislocation under ether anesthesia. Thoracic aorta was excised and cleaned from the fibroadipose tissue and from blood. Then it was rinsed with serum free M199 medium and was cross sectioned into 1-2 mm thick rings.

Preparation of tissue culture: Medium of the lower layer was prepared by adding fibrinogen and aprotinin to M199 basal medium at 3mg/ml and 5µg/ml, respectively. A 300µl of M199 medium were loaded into each well of a 48-well plate and one aortic ring was seeded. Then, 10µl thrombin were added to each well. This medium was incubated for an hour in 5% CO_2 incubator for two hours at 37 ^{o}C. After gel formation, each well was covered with 1 ml M199 medium supplemented with 20% fetal bovine serum, 0.1% aminocaproic acid, 1% L-glutamine (2 mM), 0.6% gentamycin and 1% amphotericin B. Then caryophyllene (1 µM, 10 µM or 100 µM) was added. Each concentration of the tested compound was applied to 4 wells. The plate was incubated again at 37°C in 5% CO_2 humidified incubator. On day 4, the upper layer of the medium was changed. Cultures were examined twice weekly with an inverted microscope for any visual evidence of

angiogenesis. Dimethylsulfoxide (DMSO) was used as a solvent for caryophyllene and was considered as a negative control. Suramin (100 µg/ml) served as a positive control.

Quantification of blood vessel outgrowth: The magnitude of blood vessel outgrowth was quantified according to the method of Nicosia *et al*. (1997). Briefly, the distance of blood vessels outgrowing from the primary tissue explants was measured on day 8 using inverted light microscope (4x). For vessel quantification, Leica Quin software package was used. The growth distance of at least 20 blood vessels per ring was measured. Blood vessels were selected at regular intervals around the rings to reduce the bias. The results were presented as mean $\pm$ SD. The following formula was used to calculate the percentage of inhibition:

Percent of blood vessel inhibition= $(1- (A_o /A)) \times 100\%$

where

A_o= Distance of blood vessel growth in the sample.

A= Distance of blood vessel growth in the control.

3.10. Effect of Caryophyllene on Fertility

3.10.1. Animals

All animal procedures were conducted in accordance with Jordanian regulations for animal experimentation and care and approved by the committee of institutional animal care and use. Adult female and male albino rats (*Rattus norvegicus*) were obtained from colony raised in the animal house at the University of Jordan. They were allowed to acclimatize to the laboratory conditions for two weeks before use. Rat food chow (Hamoudeh Company, Amman, Jordan) and water were provided *ad libitum*. Animals were maintained under

conventional laboratory conditions. Temperature was kept between 18-25°C. In the following experiments mating was monogamous (one male to one female). Every morning during the mating period, each female was examined for the presence of vaginal plugs or sperm in vaginal smears. Presence of spermatozoa in the vaginal smear was considered an evidence of mating, and the day of their occurrence was designated as day 1 of pregnancy.

3.10.2. Effect of Caryophyllene on Ovulation

Each virgin female rat received 0.9 ml of 50 mg/kg caryophyllene in 2.5% tween i.p for 3 consecutive days starting from day -3 to -1 before mating. The second group (control) received 0.9 ml 2.5% tween 20 according to the same protocol. Females (160-165 g) were then allowed to mate. On day 10, rats were laparotomized and cardiac blood was collected for hormone measurement. The implantation sites, adsorbing sites and corpora lutea were counted. Each group consisted of 6-8 rats.

3.10.3 Effect of Caryophyllene on Implantation

Mated females weighing 160-165 g were randomly divided into two groups of 6-8 rats each. In the first group, each pregnant rat received 0.9 ml of 50 mg/kg caryophyllene emulsified in 2.5% tween i.p for 3 consecutive days starting from day 3 to 5 of gestation. The second group (control) received 0.9 ml 2.5% tween 20 in a similar way. On day 10, rats were laparotomized and one milliliter of cardiac blood was collected for hormone analysis. The implantation sites, adsorbing sites and corpora lutea were counted.

3.10.4. Effect of Caryophyllene on Mid-Gestation

Each female rat (200-280 gm) received i.p. injection of caryophyllene (50 mg/kg in 2.5% tween) or 2.5% tween 20 for three consecutive days (day 13–15 of gestation). Experiments

were ended by laparotomy on day 16 under ether anesthesia. About one milliliter of cardiac blood was collected for hormone measurement. Fetuses were collected, counted and weighed. Each group consisted of 6-8 rats.

3.10.5. Effect of Caryophyllene on Late-Gestation

Timed-pregnant rats (200-280 gm) on day 18 of gestations were randomly divided into two groups of six rats each. Each pregnant rat received i.p. a daily dose of 50 mg/kg caryophyllene for 3 consecutive days starting from day 18 to 20 of gestation. The second group (control) received 1.0 ml 2.5% tween 20 according to the same protocol. On day 21 of gestation females were laparotomized under ether anesthesia. About 1ml of cardiac blood was collected for progesterone measurement. The viable, non-viable (adsorbing sites) fetuses and the number of corpora lutea for each ovary were counted. Each group consisted of 6-8 rats.

3.10.6 Effect of Caryophyllene on Normal Delivery

Timed-pregnant rats on day 18 of gestations were randomly divided into two of 7 rats each. Each pregnant rat received intraperitoneally a daily dose of 50 mg/kg caryophyllene or 2.5% tween 20 for 3 consecutive days starting from days 18 to 20 of gestation. Females were allowed to deliver spontaneously. Number of pups, their weight and the day of delivery were recorded. Each group consisted of 5-7 rats.

3.10.7. Analysis of Hormones

Progesterone and estrogen serum levels were analyzed using Roche (Germany) radio immuno assay (RIA) kits for both hormones by the licensed Sultan Central Laboratories, Amman, Jordan.

3.11. Statistical Analysis

The statistical analyses were performed using Statistical Package for Social Sciences (SPSS) version 17. The effect of treatments on cyst regression was evaluated by one-way analysis of covariance (ANCOVA) using Sidak corrected *post hoc* comparisons. For aortic ring assay and histological scoring, one-way analysis of variance (ANOVA) was used followed by least significant difference (LSD) analysis. For fertility studies, independent-t test was performed to compare control and caryophyllene-treated groups. In all tests a P value < 0.05 was considered significant.

Results

4.1. Effect of tested compounds on regression of endometrial explants

Four weeks after transplantation, the deposit (uterine implant) appeared as a transparent cystic area (Figure 14 A, B) with a fine superficial vascularization. Fat adhesions were very common (Figure 14 C). Histological examination revealed the presence of endometrial tissue containing stroma and glandular epithelium. In those animals which underwent oophorectomy after cyst development, the cyst disappeared regardless to its original size after 21 days (Figure 15).

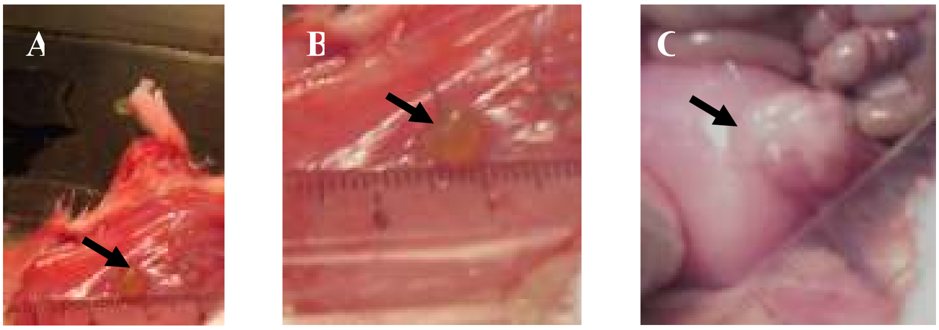


Figure 14. Growth of the endometrial implant into a cystic structure.
(A) Transparent cyst appeared at the site of transplantation (arrow). (B) Enlargement of the cyst shown in figure A (arrow). Note the presence of blue suture. (C) Fat adhesion over the cyst (arrow).

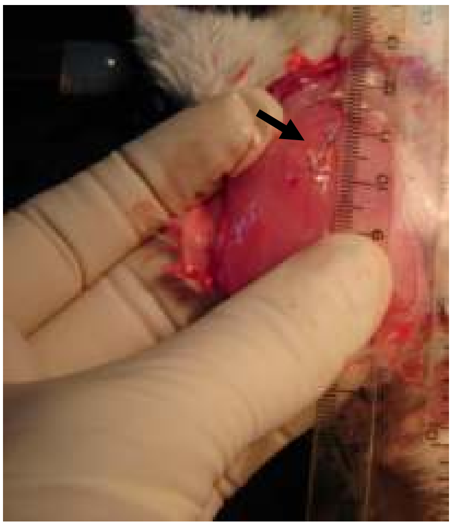


Figure 15. Disappearance of the cyst 21 days after oophorectomy (arrow).

Ten mg/kg of caryophyllene, NDGA (masoprocol), primaquine, sunitinib and theophylline reduced cyst cross sectional area by 52.5%, 45.4%, 43.1%, 80.3%, and 52.0% respectively. Forty-two µg/kg daily dose of vitamin D resulted in 51.1% reduction. Caffeine, citronellol and thymoquinone (both 1 mg/kg and 2 mg/kg doses) produced no statistically significant effect (Table 6).

Table 6. Cross-sectional area of the cyst before and after treatment (Values are Mean±SE).

Treatment	Number Of Rats/Group	Cross Sectional Area Before Treatment (mm^2)	Cross Sectional Area After Treatment (mm^2)	% Inhib-ition
Caffeine (10 mg/kg)	N=6	16.2±1.4	13.3±1.7	17.9 %
Caryophyllene (10 mg/kg)	N=8	13.9±1.1	6.6±0.6	52.5 %*
Caryophyllene (30 mg/kg)	N= 9	12.2±1.5	7.2±1.6	41.0 %*
Citronellol (10 mg/kg)	N= 8	12.4±2.9	9.5±2.2	23.4 %
NDGA (Masoprocol) (10 mg/kg)	N=7	13.0 ±1.6	7.1±1.1	45.4 %*
Primaquine (10 mg/kg)	N=13	15.3 +2.6	8.7+ 1.3	43.1 %*
Sunitinib (10 mg/kg)	N=9	12.7±1.1	2.5±0.9	80.3 %*
Theophylline (10 mg/kg)	N=7	17.3 ± 1.8	8.3 ± 2.3	52.0 %*
Theophylline (15 mg/kg)	N=7	14.8±3.0	5.1±1.2	65.5 %*
Thymoquinone (1 mg/kg)	N= 6	15.5±2.1	17.4±2.2	-12.3 %
Thymoquinone (2 mg/kg)	N=8, 2 died after 19 days of treatment	13.0±2.0	15.7±1.6	-20.8 %
Vitamin D (42 µg/kg)	N=9	13.3±2.1	6.5±1.4	51.1 %*
Oophorectomy (OVX)	N=7	14.6±4.1	0±0	100 %*
Negative control (DMSO)	N= 7	12.8 ±1.6	11.4±1.1	10.9 %
Negative control (0.85% NaCl)	N= 9	17.9 ±2.7	16.2±1.5	10.6 %
Negative control (2.5% tween 20)	N= 10	11.8±1.6	11.5±1.5	2.5 %

* Statistically significant difference from its control, $P<0.05$

Ten mg/kg sunitinib given daily for 21 days resulted in complete cyst disappearance in 50% of the animals. Furthermore, fat adhesions were almost absent (Figure 16A). However, in those animals treated with sunitinib interference with surgical wound healing was noticed. Also, yellow coloration of hair and urine was observed (Figure 17).

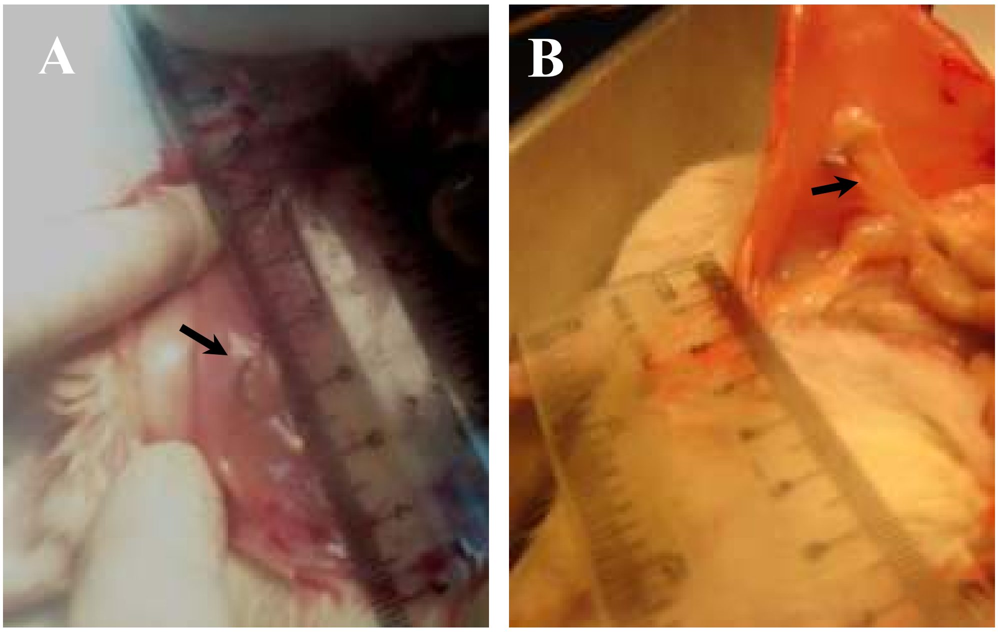


Figure 16. Cyst appearance after treatment with sunitinib (A) and with caryophyllene (B) (A) Disappearance of the cyst after treatment with sunitinib (arrow). Note the absence of adhesions (B) Fat adhesion in an animal treated with 30 mg/Kg caryophyllene (arrow).

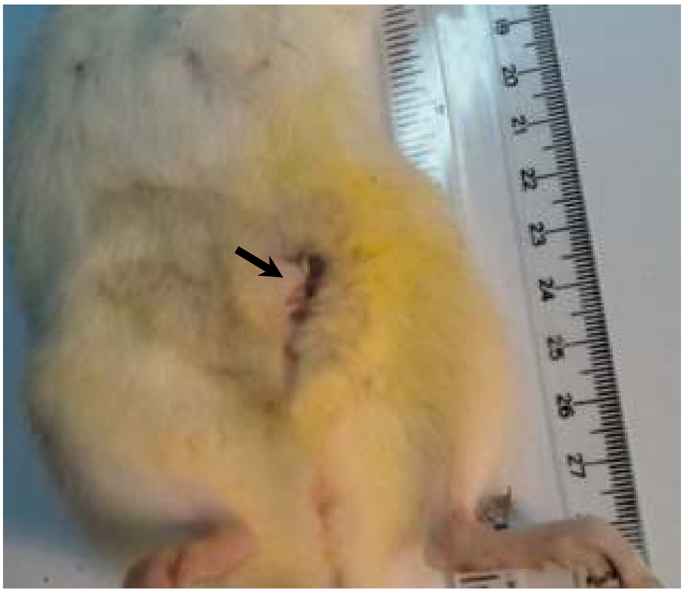


Figure 17. Sunitinib interfered with healing of the surgical wound. The arrow points at the unhealed surgical wound 21 days after the second surgery. Note the yellow coloration of hair.



Figure 18. Reduction of the cyst size after treatment with theophylline (15 mg/kg) (arrow).

4.2. Histological Findings

4.2.1. Control Group

The histological analysis of the endometrial cysts revealed that they totally adhered to the abdominal musculature (Figure 19 A). The epithelial layer (luminal epithelium) was well-preserved (Figure 19 B). Smooth muscles were present only at the side of skeletal muscles, not around the whole cyst. These smooth muscles are the ones which were present originally in the endometrial fragment cultured in the first surgery.

In animals which received the vehicle as the only treatment, glandular tissue was present within stroma (Figure 20 A). The stroma was highly vascularized especially around glandular tissue, heavily infiltrated with inflammatory cells. Cyst lumen contained mixed inflammatory cells and sometimes red blood cells. Also, inflammatory cells can be seen within the lumen of glandular tissue (Figure 20 A).

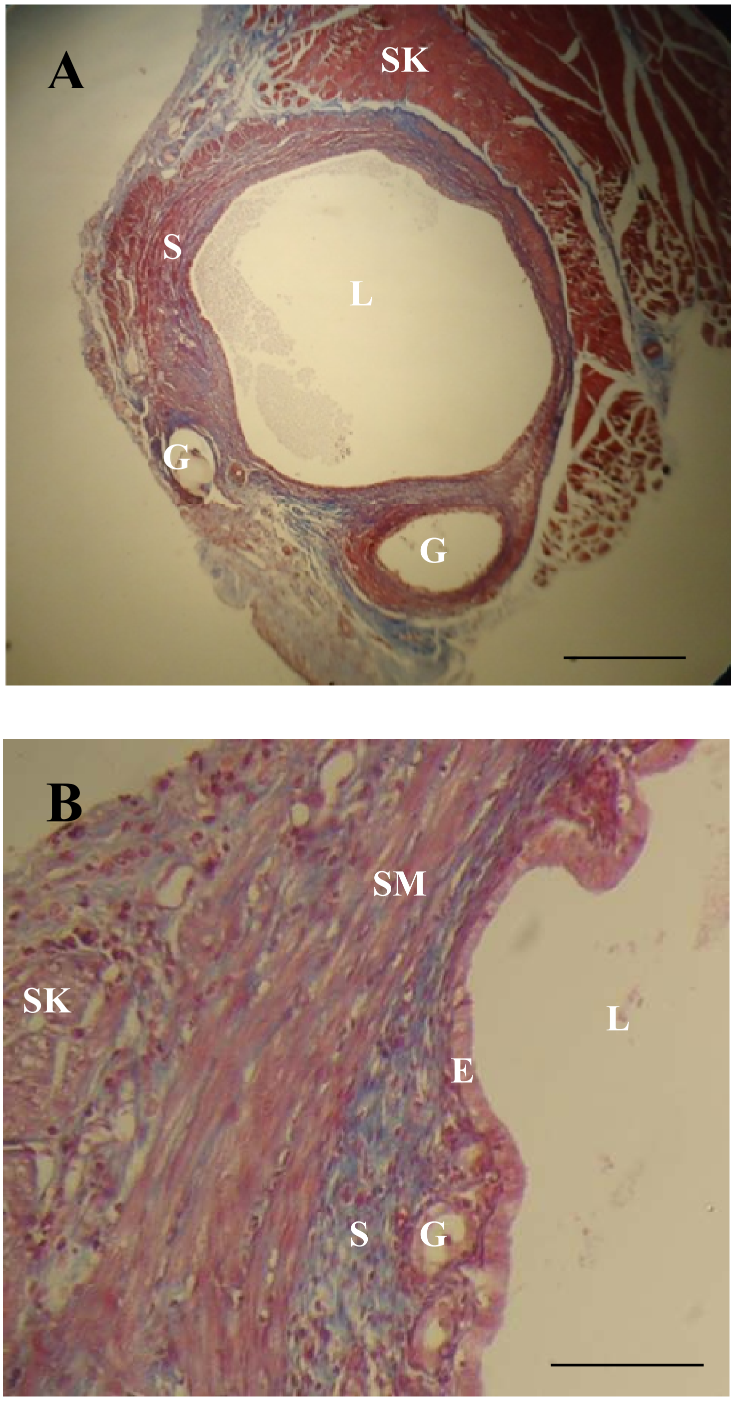


Figure 19. Light microscopic picture of an endometrial cyst.
(A) This figure shows a cyst with lumen (L), lined with luminal epithelium (E), and stroma (S) containing glandular tissue (G). (B) Enlargement of the lining of the cyst lumen. Smooth muscles (SM), which were present originally in the endometrial fragment cultured in the first surgery, are still present. Note the adherence of the cyst to skeletal muscles (SK) (Masson trichrome stain). Scale bar=100 µm in A & B.

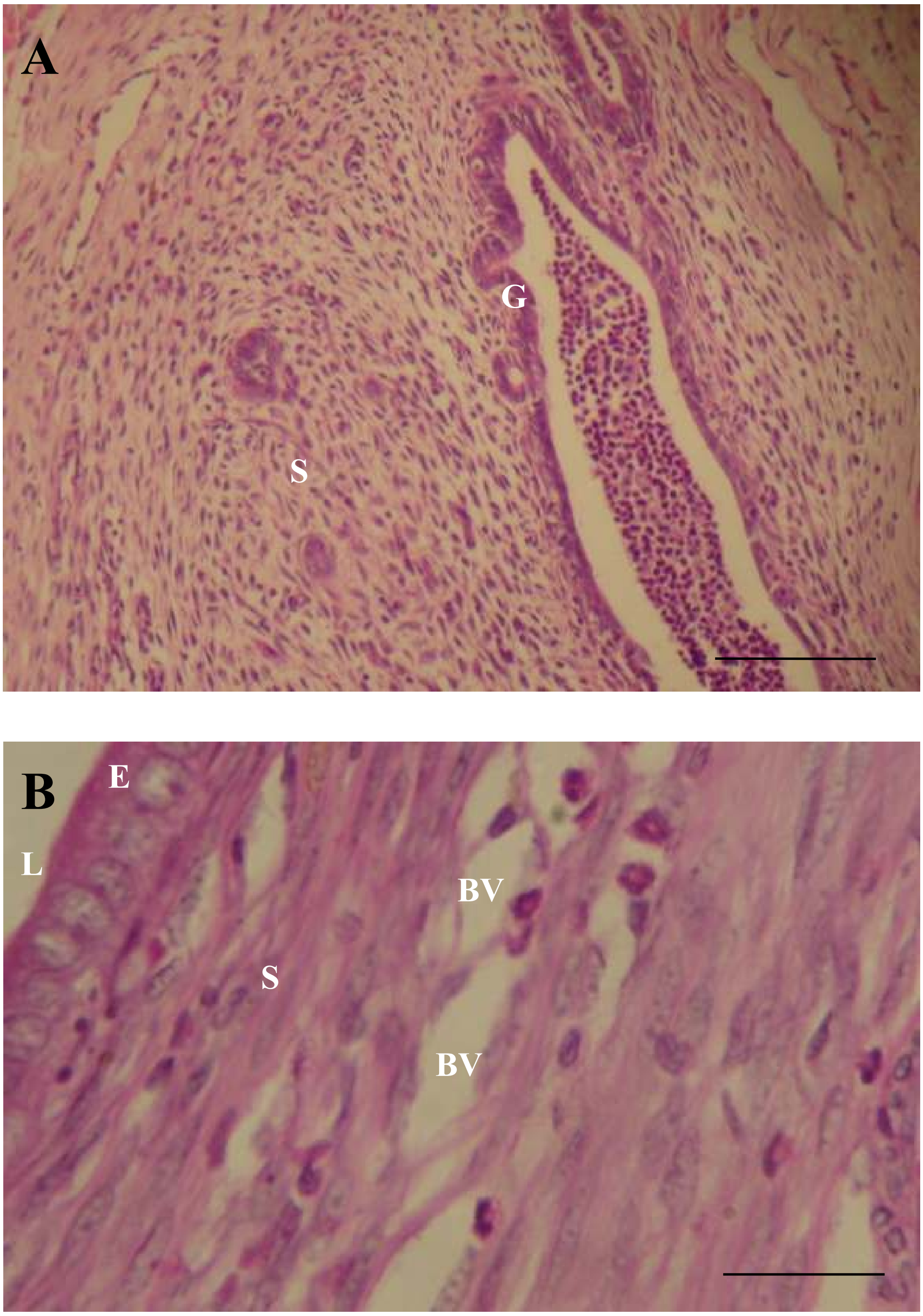


Figure 20. Light microscopic picture of a control rat.
(A) The stroma contains glandular tissue rich with inflammatory cells in its lumen. (B) Luminal epithelium was well preserved in the control-group. The stroma was higly vascularized (H& E stain). BV: blood vessel; E: luminal epithelium; G: glandular tissue; L: cyst lumen; S: stroma. Scale bar=100µm in A; 25µm in B.

Despite not being visible to the naked eye, endometrial tissue was detected microscopically in oophorectomized rats. Degenerating glandular tissue was present within stroma but highly reduced. Also, the stroma was highly vascular (Figure 21).

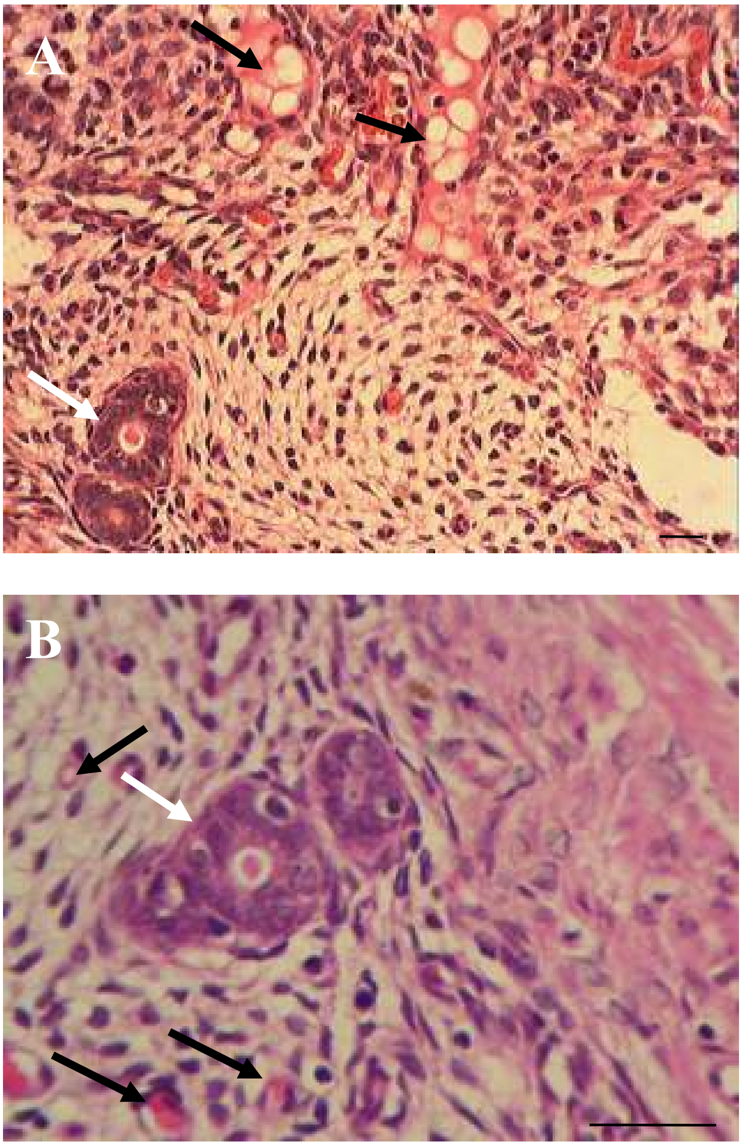


Figure 21. Light microscopic picture of a cyst of an oophorectomized rat. (A) Glandular tissue (white arrow) is present within stroma but highly reduced. Note the presence of spiral blood vessels (black arrows). (B) Degenerative changes within glandular tissue (white arrow) are evident. Note the high number of blood vessels around the glandular tissue (black arrows) (H& E stain). Scale bar=25µm in A & B.

4.2.2. β-Caryophyllene-treated group

Histological examination of caryophyllene-treated rats (both 10 mg/kg and 30 mg/kg doses) revealed that the cyst was reduced in size. In most specimens, stroma was thinner than that of the control. Glandular tissue within stroma was almost absent (Figure 22 A, B). The stroma was infiltrated with inflammatory cells including eosinophils, lymphocytes and hemosiderin-laden macrophages (Figure 23). In cyst lumen, mixed inflammatory cells were seen. Fibrosis was mild and focal in the stroma.

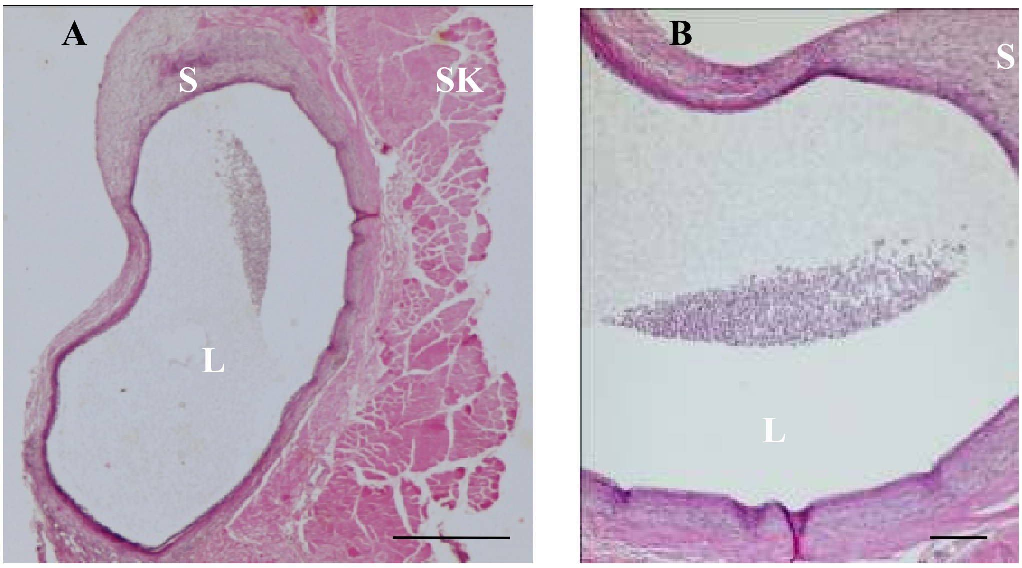


Figure 22. Glandular tissue within stroma of cysts in β-caryophyllene-treated rats was scarce. (A&B) Glandular tissue is not present within stroma. Note the presence of inflammatory cells within cyst lumen (H&E stain). L: cyst lumen; S: stroma; SK: skeletal muscles. Scale bar= 1000µm in A, 250 µm in B.

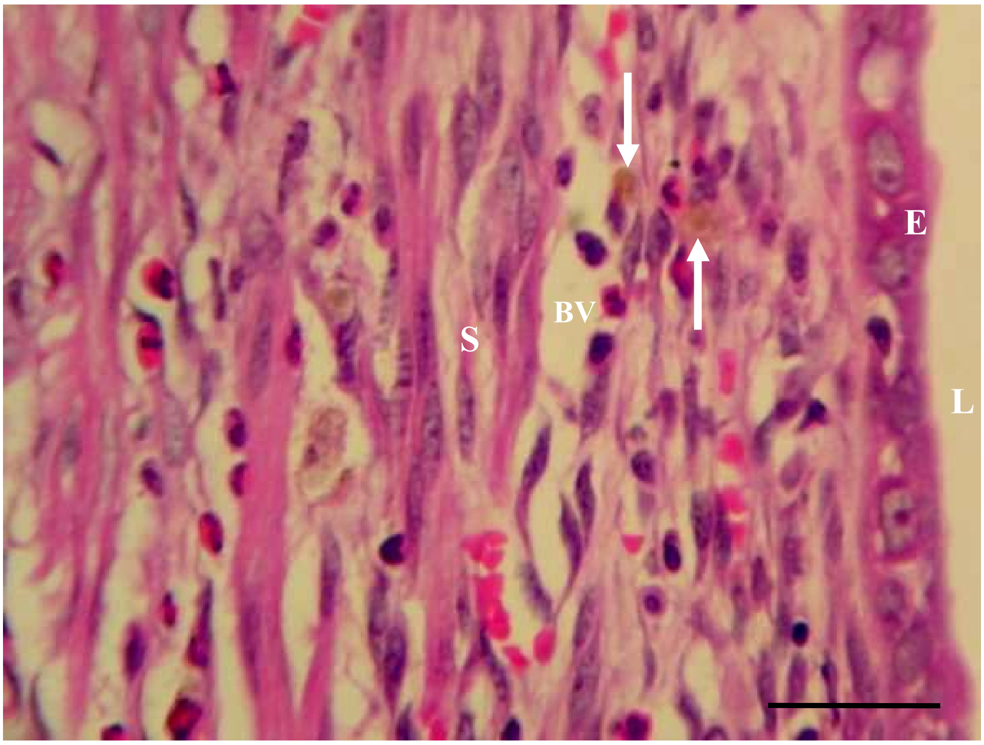


Figure 23. Light microscopic picture of a cyst of a β-caryophyllene-treated rat. The stroma is infiltrated with mixed inflammatory cells. Note the presence of hemosiderin (white arrows) in the stroma and leukocytes in the lumen of blood vessels. Hemosiderin is usually present in healed foci of endometriosis (H& E stain). BV: blood vessel; E: luminal epithelium; L: cyst lumen; S: stroma. Scale bar= 25 µm.

4.2.3. Masoprocol-treated group

Degenerative changes in luminal epithelium were evident in masoprocol-treated group (Figure 24 A, B). Glandular tissue within stroma was scarce. Mild fibrosis was found within stroma localized directly under the luminal epithelium (Figure 25 A, B). The stroma was infiltrated by eosinophils and hemosiderin-laden macrophages and other inflammatory cells. Inflammatory cells were seen also in cyst lumen (Figure 26).

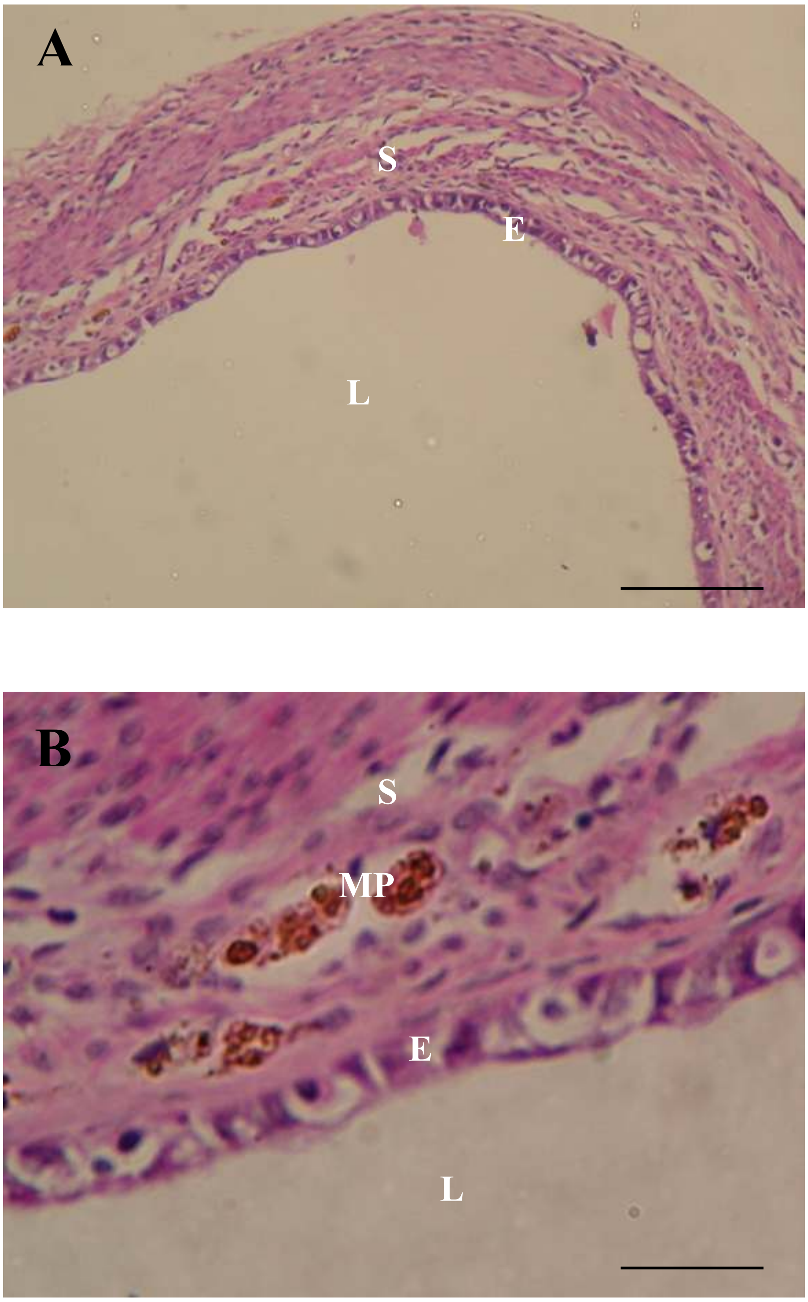


Figure 24. Degenerative changes in luminal epithelium of a cyst of a masoprocol-treated rat. Note the absence of glandular tissue in A and the presence of hemosiderin-laden macrophages in B. Hemosiderin is usually present in healed foci of endometriosis (H& E stain). E: luminal epithelium; L: cyst lumen; MP: hemosiderin-laden macrophages; S: stroma. Scale bar =100 µm in A; 25 µm in B.

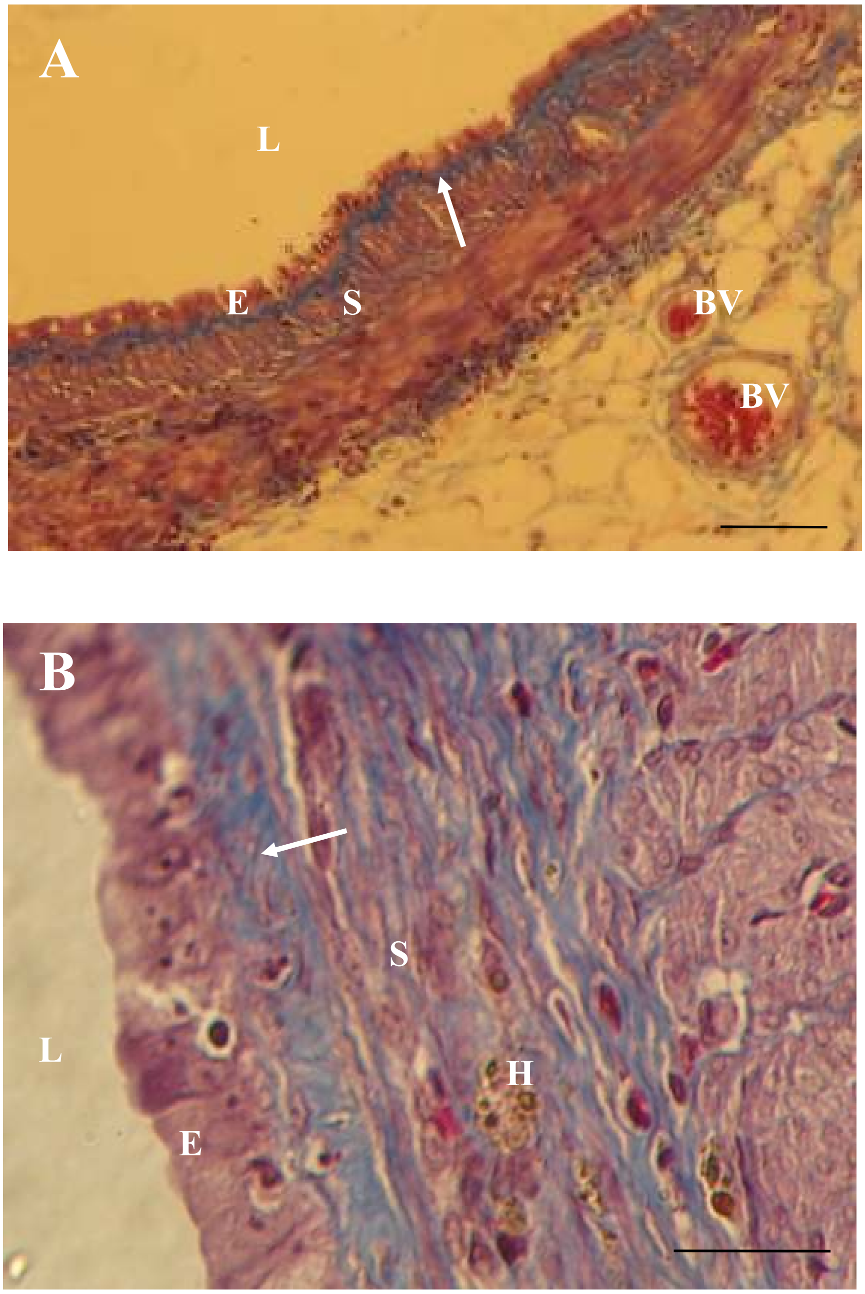


Figure 25. Fibrosis in the stroma of a cyst of a masoprocol-treated rat.
A & B Fibrosis was noticed within stroma (blue color). It was localized mainly under the luminal epithelium (white arrows) (Masson trichrome stain). BV: blood vessel; E: luminal epithelium; H: hemosiderin; L: cyst lumen; S: stroma. Scale bar =1000µm in A; 25µm in B.

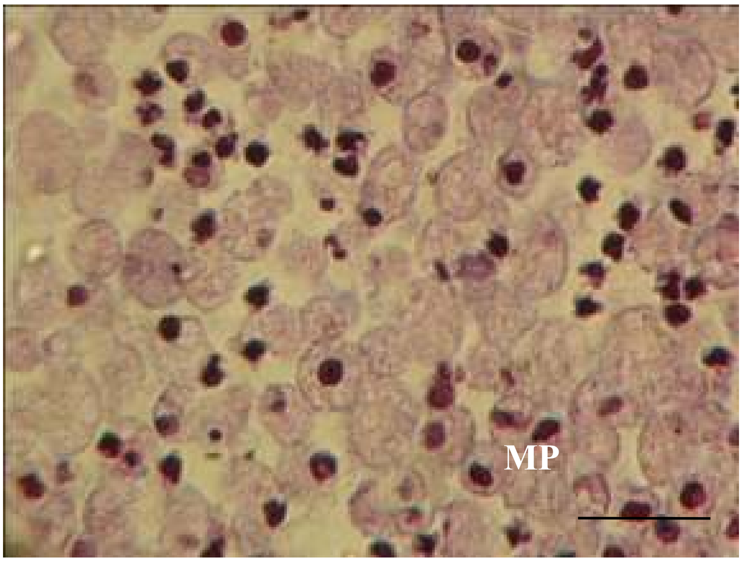


Figure 26. Inflammatory cells within the lumen of a cyst of a masoprocol-treated rat. Predominance of macrophages in the lumen of the cyst (H& E stain). MP: macrophages. Scale bar =25µm.

4.2.4. Primaquine-treated group

Degenerative changes and apoptotic bodies were seen in luminal epithelium (Figure 27 A). However, glandular tissue within stroma was present (Figure 27 A). Mixed inflammatory cells in cyst lumen were noticed (Figure 27 B). Fibrosis (Figure 28), eosinophilic infiltrate and hemosiderin-laden macrophages were also present in stroma.

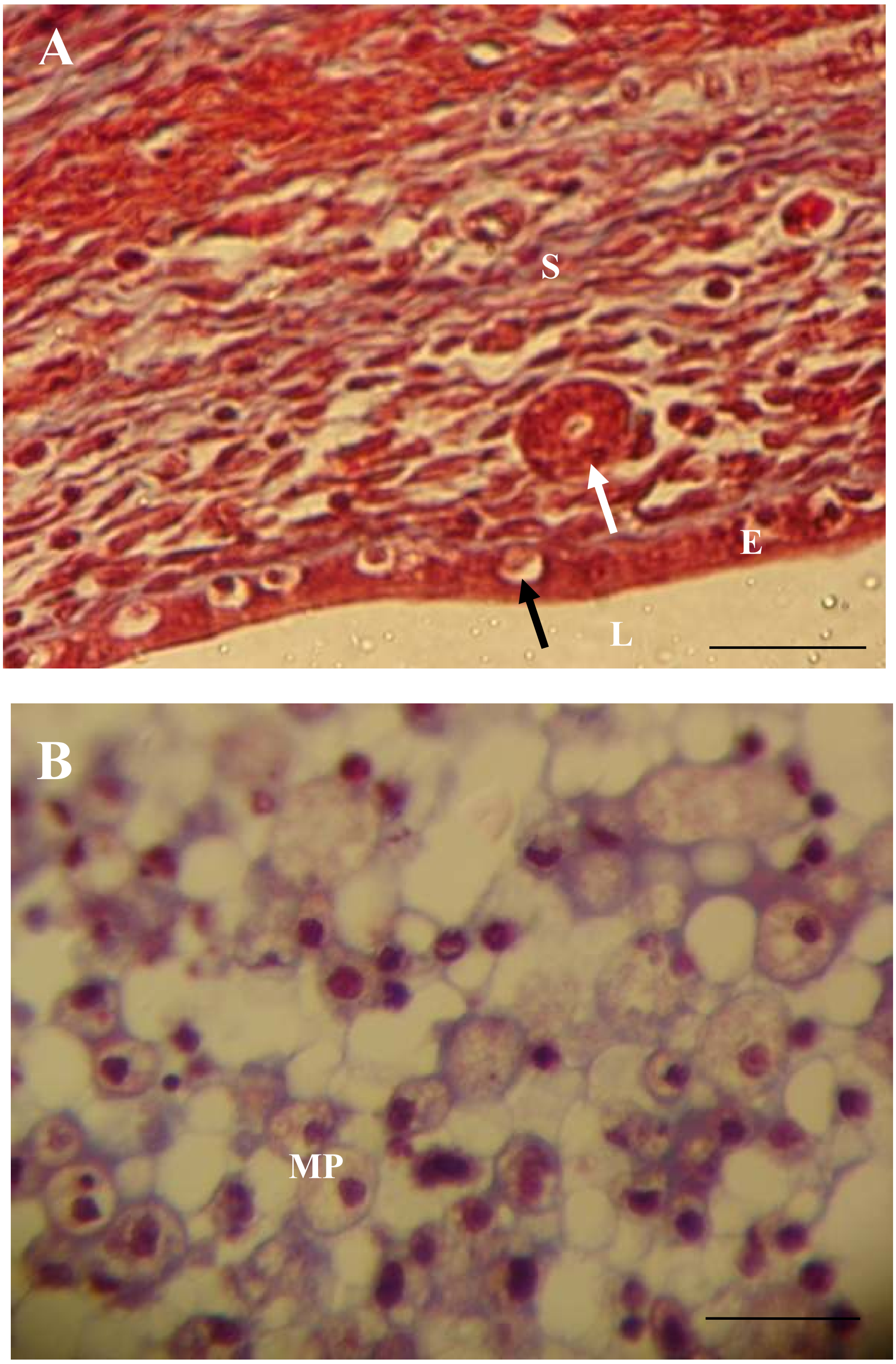


Figure 27. Light microscopic picture of a cyst of a primaquine-treated rat. (A) Degenerative changes in luminal epithelium (black arrow). Note the presence of reduced glandular tissue (white arrow). (B) Inflammatory cells in cyst lumen (H& E stain). E: luminal epithelium; L: cyst lumen; MP: macrophages; S: stroma. Scale bar=25 µm in A& B.

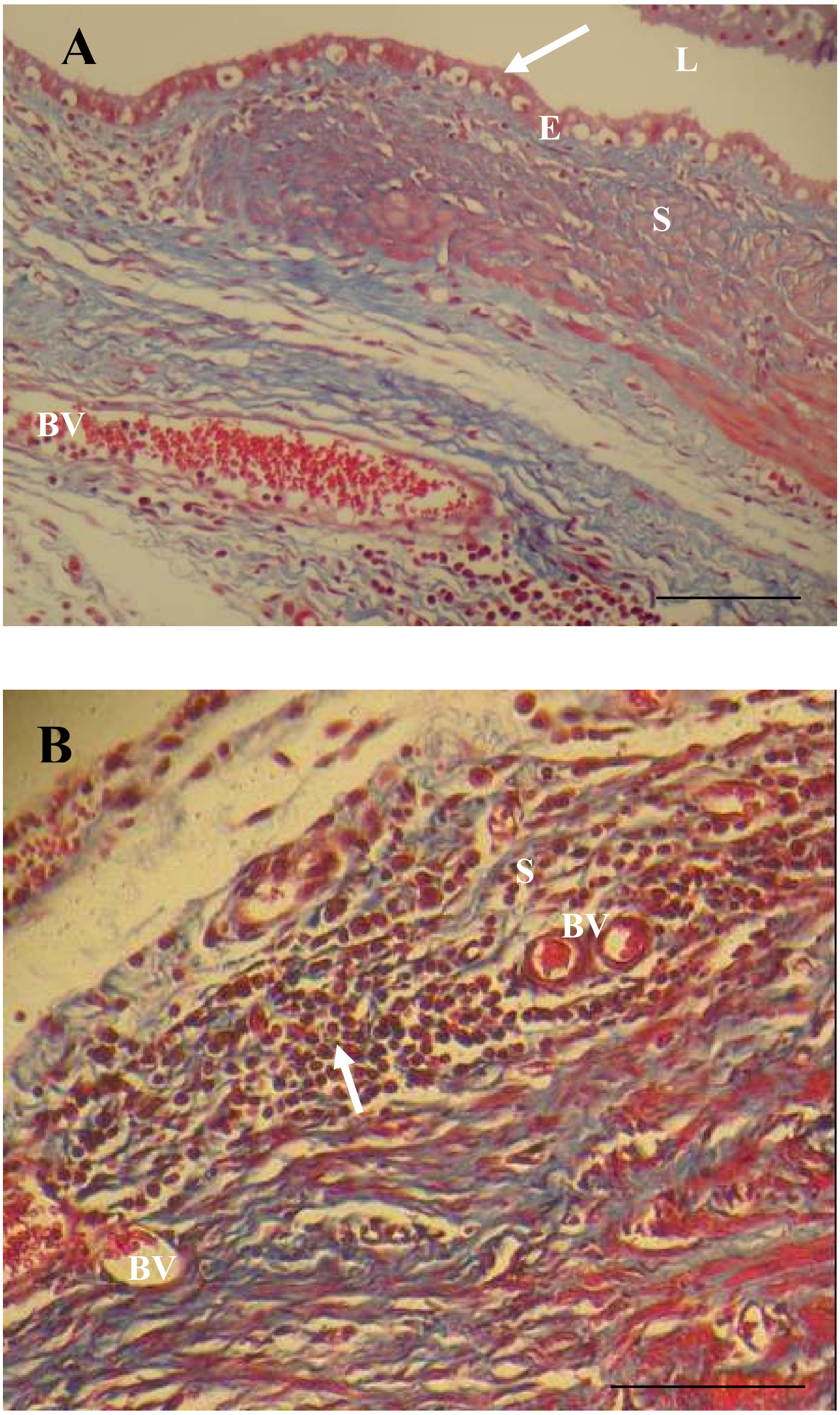


Figure 28. Fibrosis in stroma of a primaquine-treated rat. (A) Fibrosis was found within stroma (blue color). Note degenerative changes in luminal epithelium (arrow). (B) Heavy infiltration with inflammatory cells in stroma (arrow) (Masson trichrome stain). BV: blood vessel; E: luminal epithelium; L: cyst lumen; S: stroma. Scale bar=100µm in A& B.

4.2.5. Sunitinib-treated group

Although many cysts disappeared macroscopically in sunitinib-treated group, they were detected microscopically. However, they were greatly reduced in size. Degenerative changes in luminal epithelium were prominent (Figure 29). Also, extensive fibrosis was observed in both light (Figure 30 A, B) and electron microscopic investigations (Figure 50, 51). Glandular tissue was rarely seen within stroma. The stroma was infiltrated by inflammatory cells including eosinophils. Hemosiderin-laden macrophages were abundant (Figure 31 B).

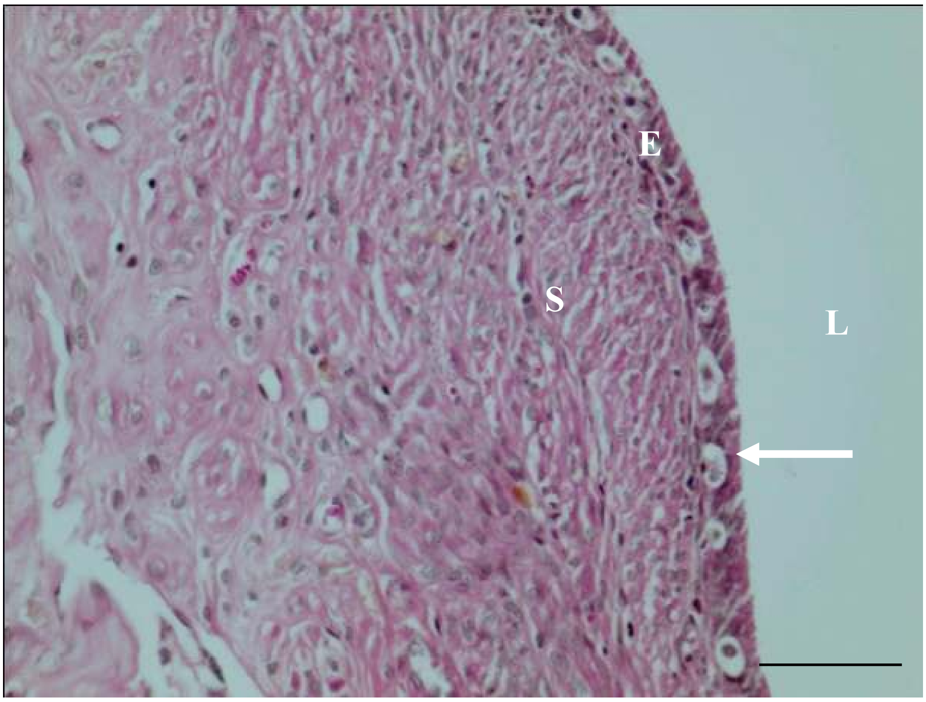


Figure 29. Degenerative changes in luminal epithelium of a cyst of a sunitinib-treated rat (arrow) (H&E stain). E: luminal epithelium; L: cyst lumen; S: stroma. Scale bar=100µm.

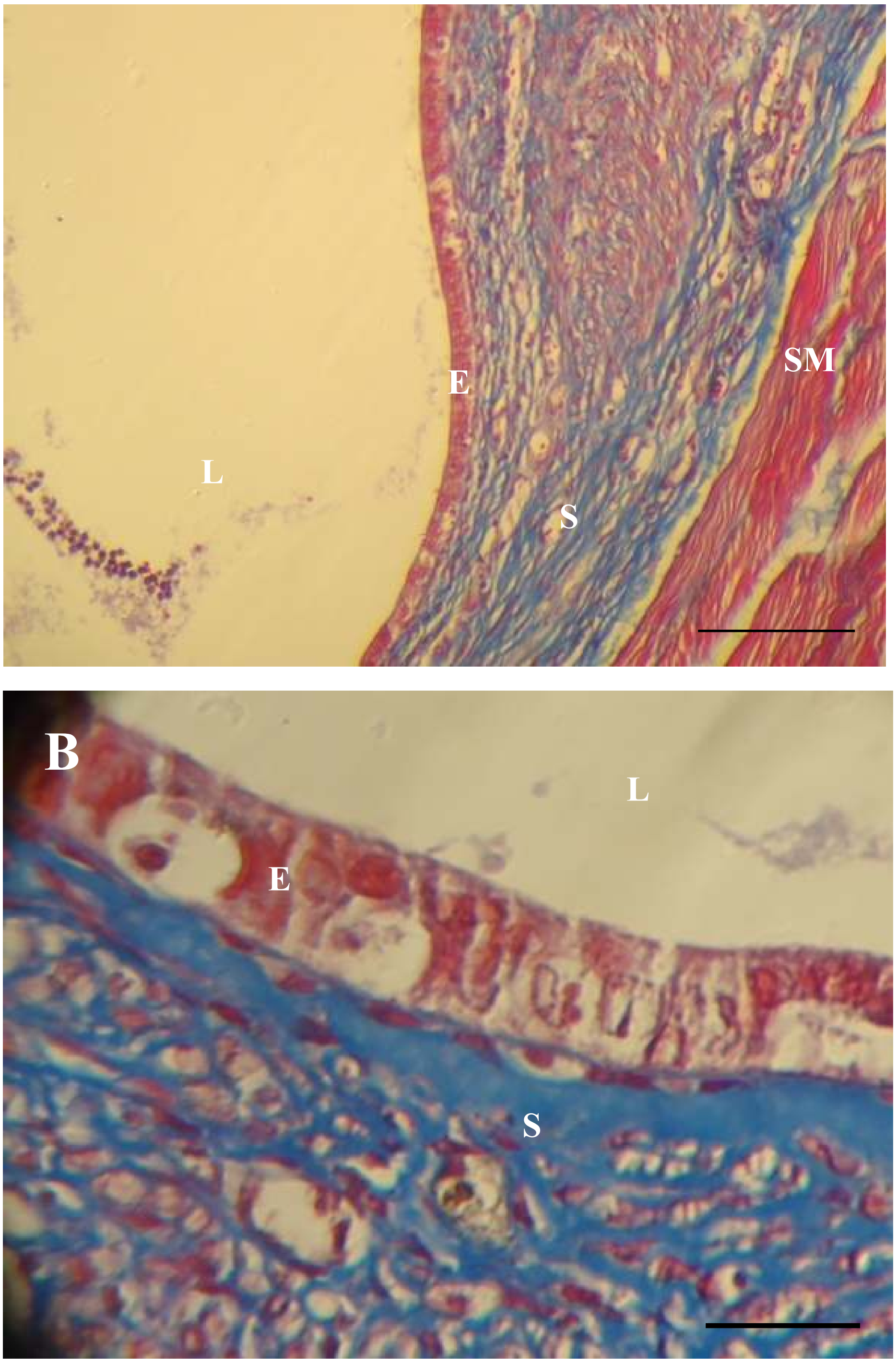


Figure 30. Light microscopic picture of a cyst of a sunitinib-treated rat.
(A, B) Extensive fibrosis (blue color) was seen within stroma in sunitinib-treated rats (Masson trichrome stain). E: luminal epithelium; L: cyst lumen; S: stroma; SM: smooth muscles. Scale bar=100µm in A; 25µm in B.

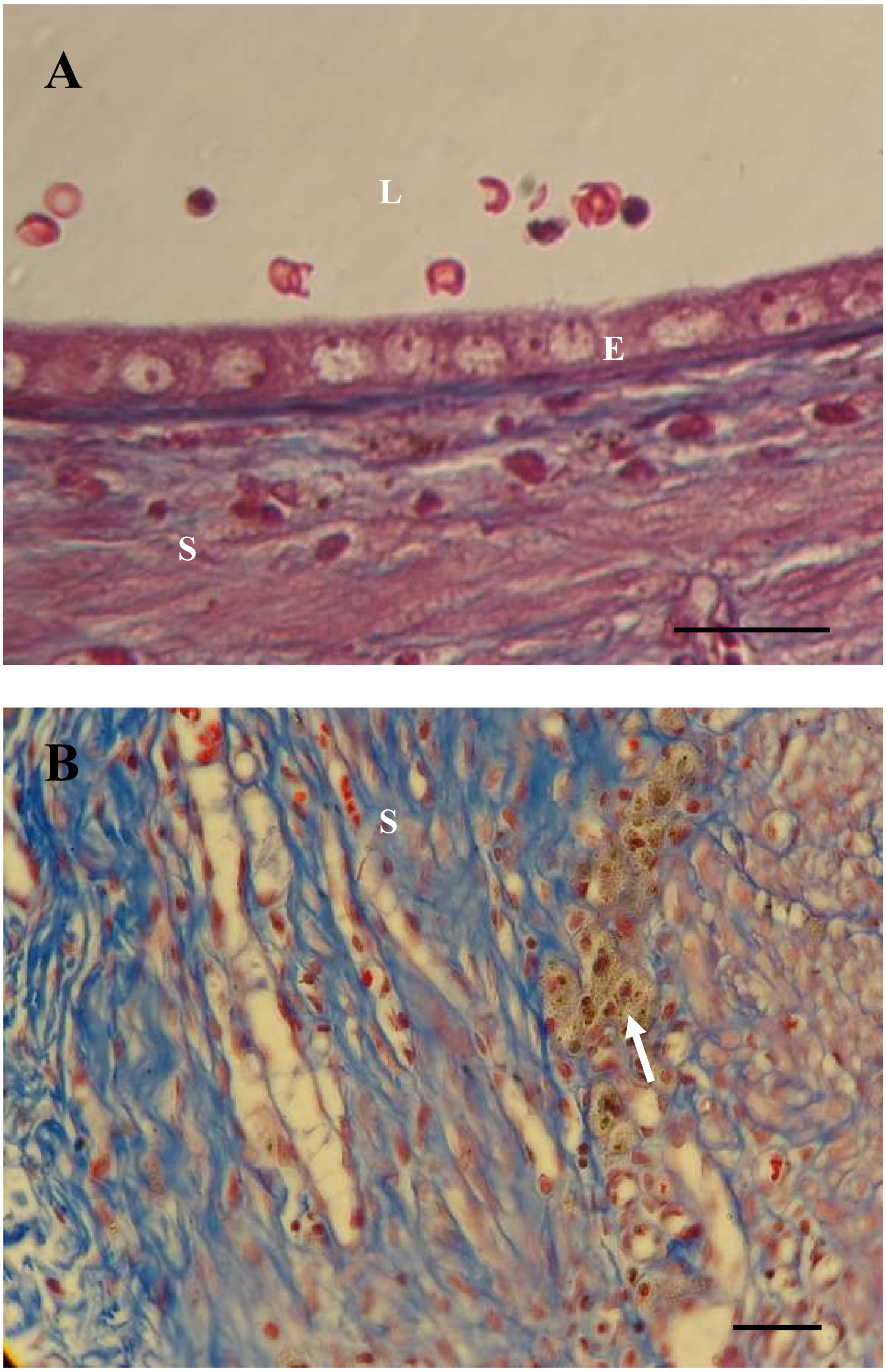


Figure 31. Extensive fibrosis was present within stroma in a sunitinib-treated rat (B) but not in control rat (A). (A) RBC are present in the lumen of the cyst of the control. (B) Hemosiderin is seen (arrow) which is usually present in healed foci of endometriosis (Masson trichrome stain). E: luminal epithelium; L: cyst lumen; S: stroma. Scale bar=25µm in A& B.

4.2.6. Theophylline-treated group

The epithelial layer was poorly preserved in some specimens of theophylline-treated group (Figure 33 A, B). Glandular tissue within stroma was scarce. The stroma was infiltrated with mixed inflammatory cells (Figure 32). Fibrosis was localized directly under the luminal epithelium (Figure 34 A, B). Mixed inflammatory cells were seen in cyst lumen.

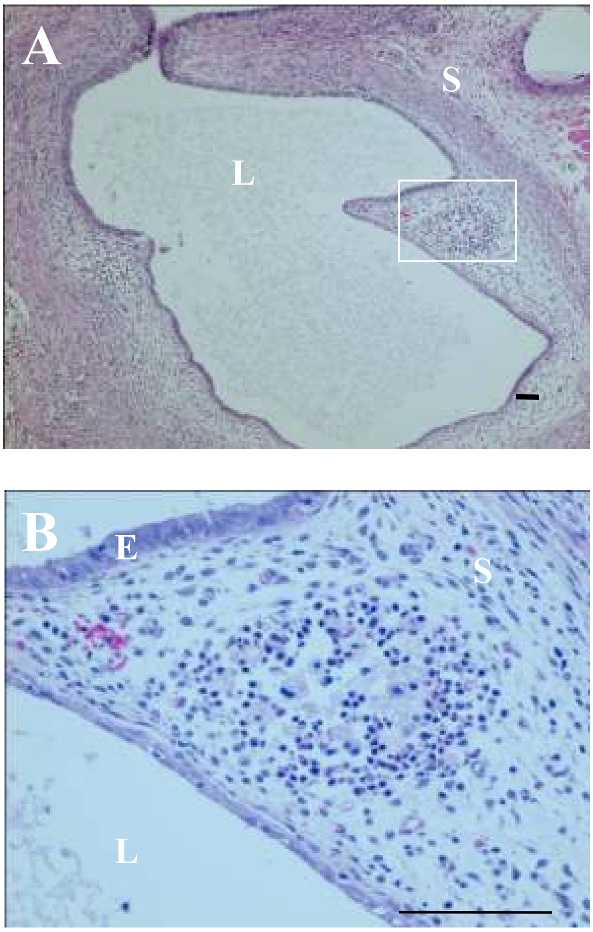


Figure 32. Heavy infiltration with mixed inflammatory cells in the stroma of a cyst of a theophylline-treated rat (A, B). Figure B shows an enlargement of the inset area in A (H& E stain). E: luminal epithelium; L: cyst lumen; S: stroma. Scale bar=100µm in A; 25µm in B.

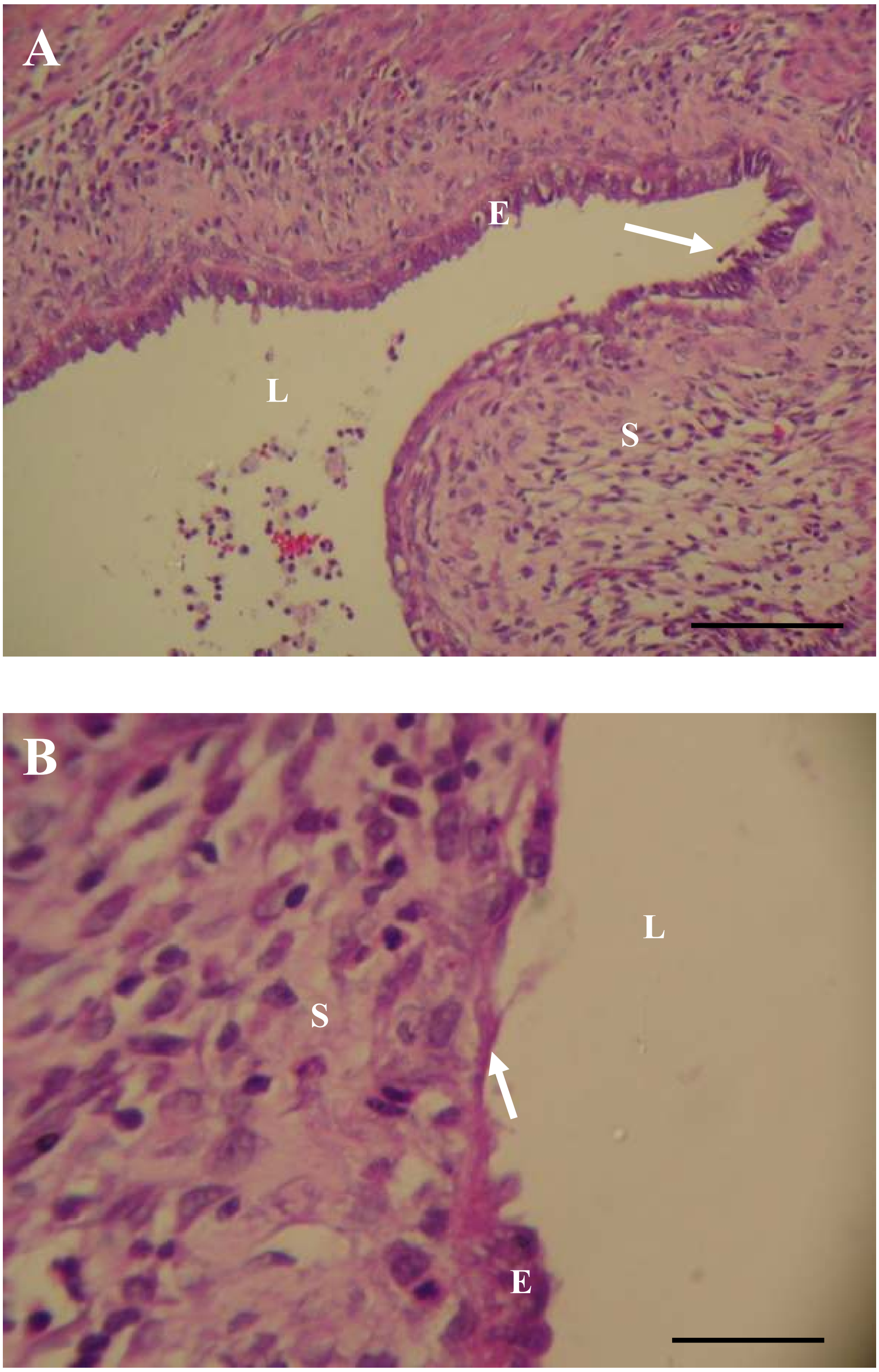


Figure 33. Light microscopic picture of a cyst of a theophylline-treated rat. Poorly preserved epithelial layer (arrow) (A, B) which is a sign of regression in endometriotic cysts (H& E stain). E: luminal epithelium; L: cyst lumen; S: stroma. Scale bar=100µm in A; 25µm in B.

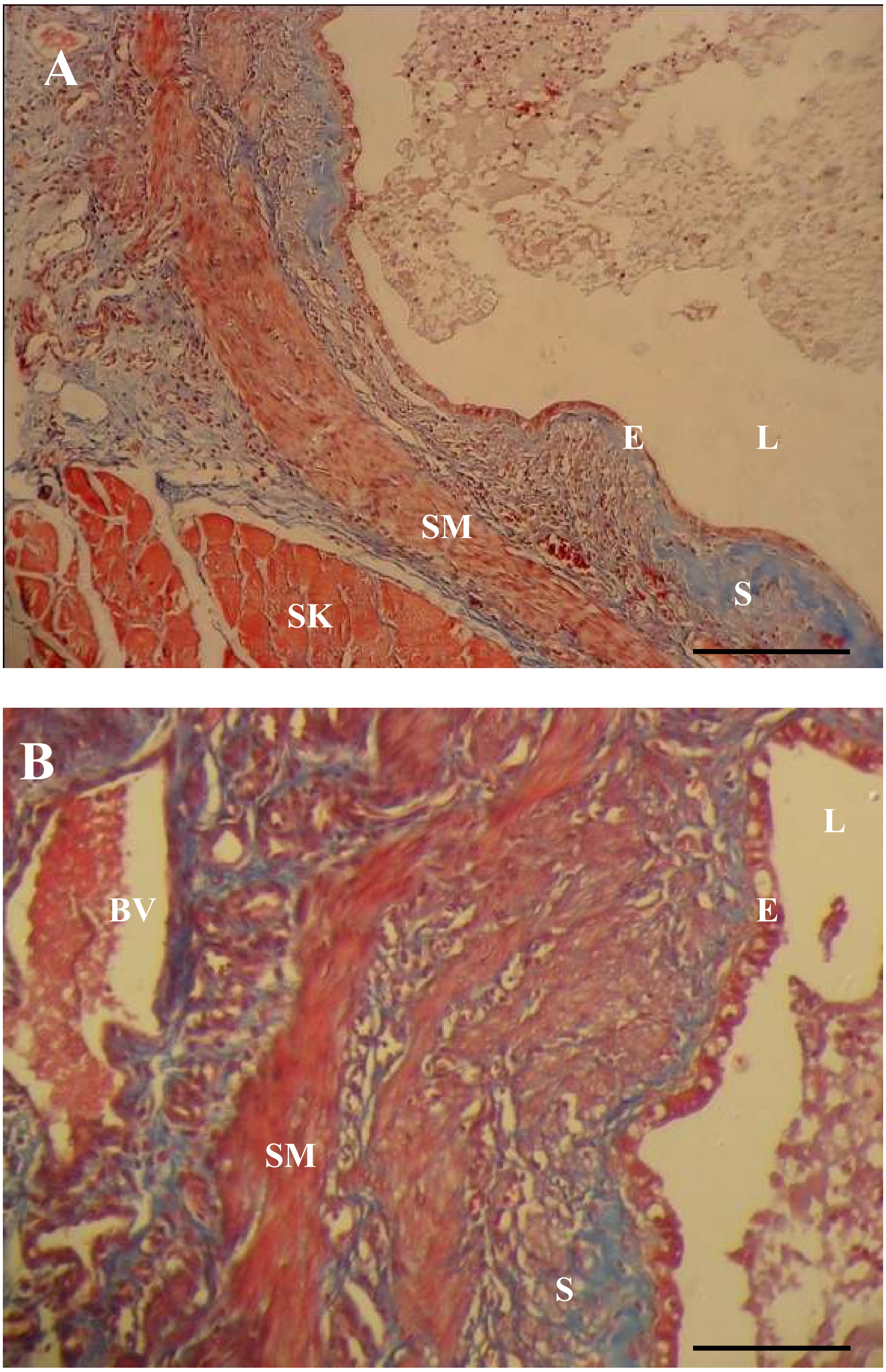


Figure 34. Fibrosis in stroma of a theophylline-treated rat. (A & B) Fibrosis was present mainly under luminal epithelium (blue color). Fibrosis is usually present in healed foci of endometriosis. Muscles are stained with red color. (Masson Trichrome stain). BV: blood vessel; E: luminal epithelium; L: cyst lumen; S: stroma; SK: skeletal muscles; SM: smooth muscles. Scale bar=100µm in A, 25µm in B.

4.2.7. Vitamin D-treated group

Glandular tissue within stroma was scarce in vitamin-D treated group. The stroma was infiltrated with few inflammatory cells including eosinophils and hemosiderin-laden macrophages (Figure 35). Cyst lumen contained lymphocytes and abundance of macrophages. Fibrosis was seen as a thin ring directly under luminal epithelium (Figure 36).

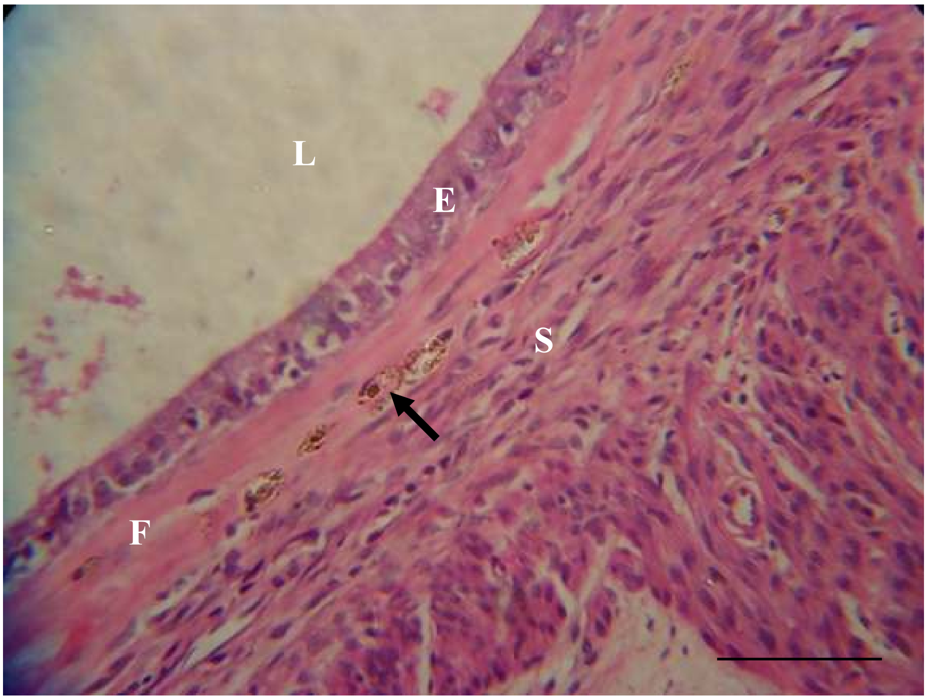


Figure 35. Light microscopic picture of a cyst of a vitamin D-treated rat. Fibrosis and hemosiderin-laden macrophages are seen in this section (arrow). Both are positive signs of healing process (H&E stain). E: luminal epithelium; F: fibrosis; L: cyst lumen; S: stroma. Scale bar=25µm in A.

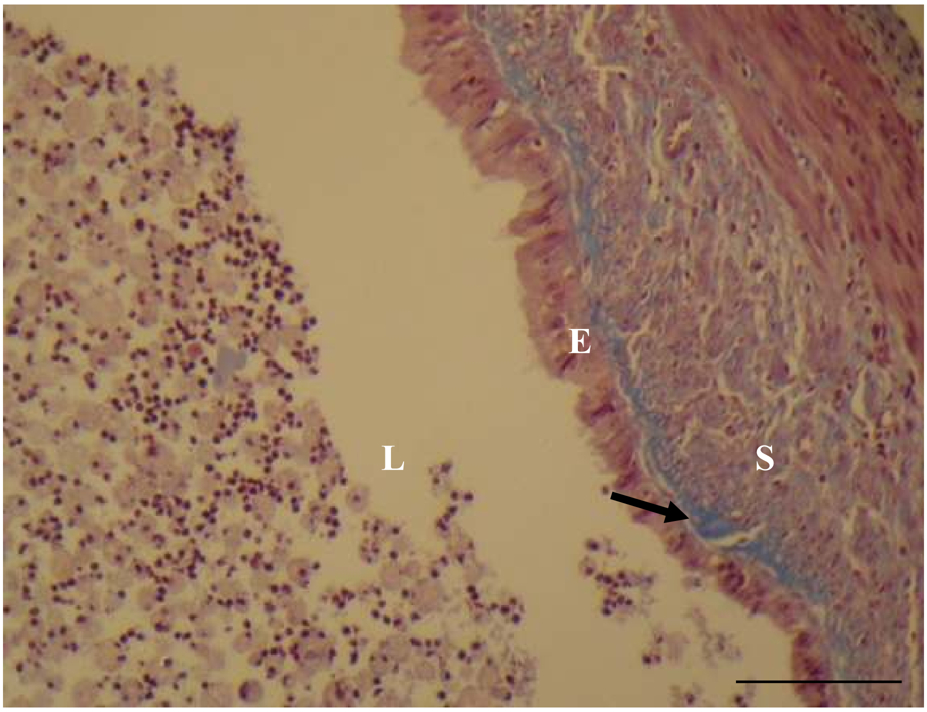


Figure 36. Fibrosis in the stroma of a cyst of a vitamin D-treated rat. Fibrosis was concentrated directly under luminal epithelium and appears as a blue line (arrow). Fibrosis is usually present in healed foci of endometriosis (Masson trichrome stain). E: luminal epithelium; L: cyst lumen; S: stroma. Scale bar=100µm.

A statistically significant reduction in the pathological score for stroma was produced by caryophyllene, masoprocol, sunitinib and vitamin D. On the other hand, no statistically significant reduction was noticed in primaquine and theophylline-treated groups (Table 7).

Table 7. Pathological scores of the tested compounds

Compound	Score (mean ± SD)
Control	2.7±0.3
Caryophyllene	1.7±0.4 *
Masoprocol	1.2±0.4 *
Primaquine	2.4±0.5
Sunitinib	0.8±0.4 *
Theophylline	2.5±0.1
Vitamin D	1.9±0.4 *

* Statistically significant different from the control, $p < 0.05$

Mast cells were mainly present at the border between stroma and adjacent tissues specifically at the highly vascularized areas (Figure 37). It was noticed that mast cells take different shapes (rounded and elongated) and sizes (small and large) in the same specimen. Degranulated mast cells were observed in specimens of animals received different treatments (Figure 38).

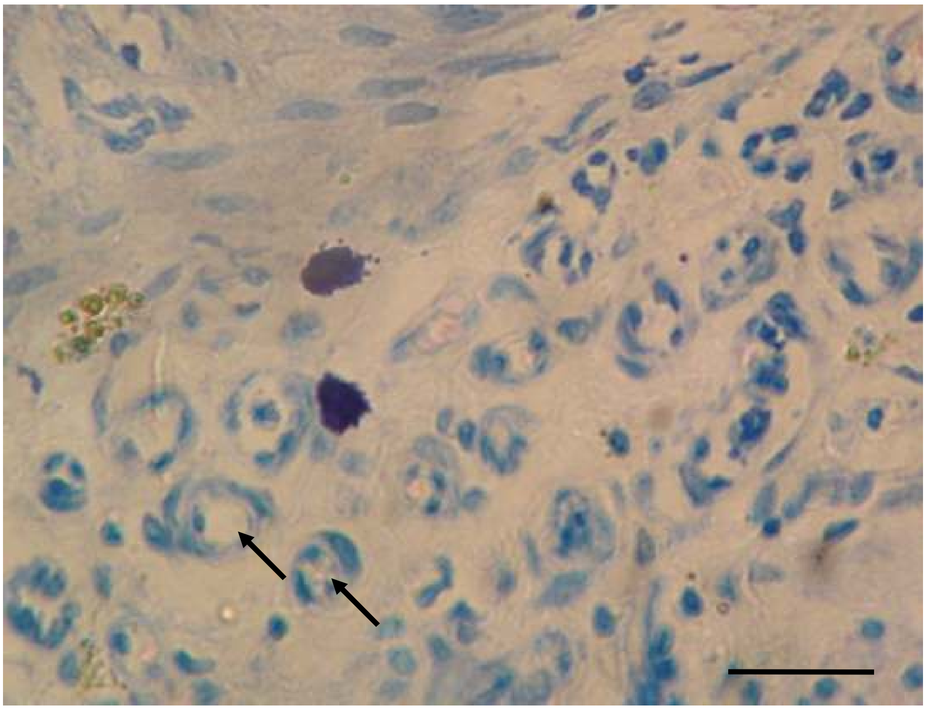


Figure 37. Light microscopic picture of mast cells of a control rat. This figure of the periphery of a cyst of a control rat shows that mast cells are present in close proximity to blood vessels (arrows) (Toluidine blue stain). Scale bar = 30μm.

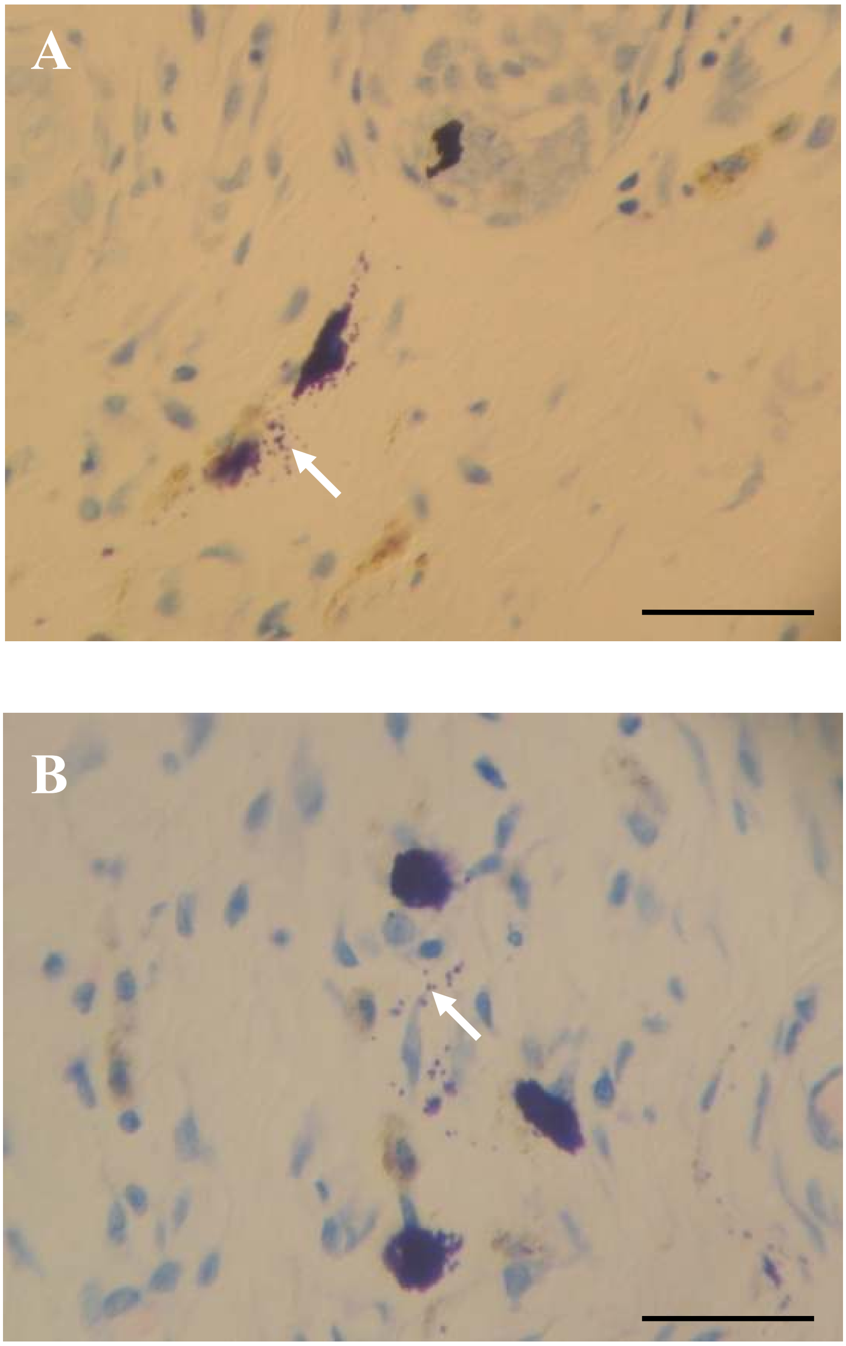


Figure 38. Degranualtion of mast cells of an endometrial cyst
(A) Caryophyllene-treated rat. (B)Vitamin D-treated rat. Granules of mast cells are present outside the cell (arrows) which indicates secretory activity of these cells (Toluidine blue stain). Scale bar = 30µm.

4.3. TUNEL assay

For the control group, TUNEL assay showed that occasional cells in luminal epithelium and stroma were apoptotic (Figure 39). For caryophyllene-treated group, most cells in cyst lining were positive as indicated by the staining of the nuclei with dark brown color (Figure 40 A). Some cells in stroma were positive as well. Interestingly, endothelial lining of some small blood vessels was also positive (Figure 40 B).

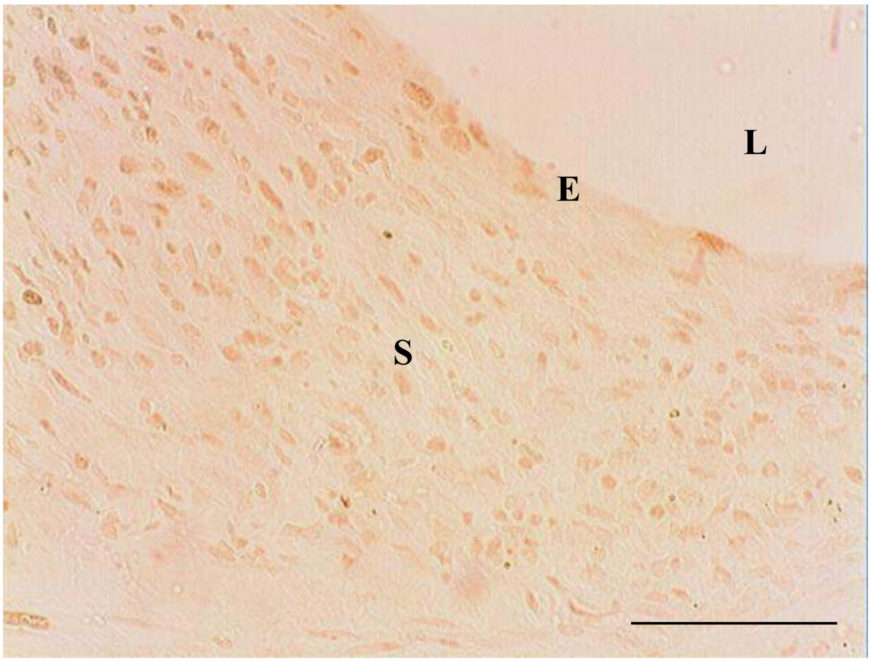


Figure 39. TUNEL assay for a control rat. The cells in this section failed to take the dark brown color in TUNEL assay which indicates a negative result for apoptosis. E: luminal epithelium; L: cyst lumen; S: stroma. Scale bar=100µm.

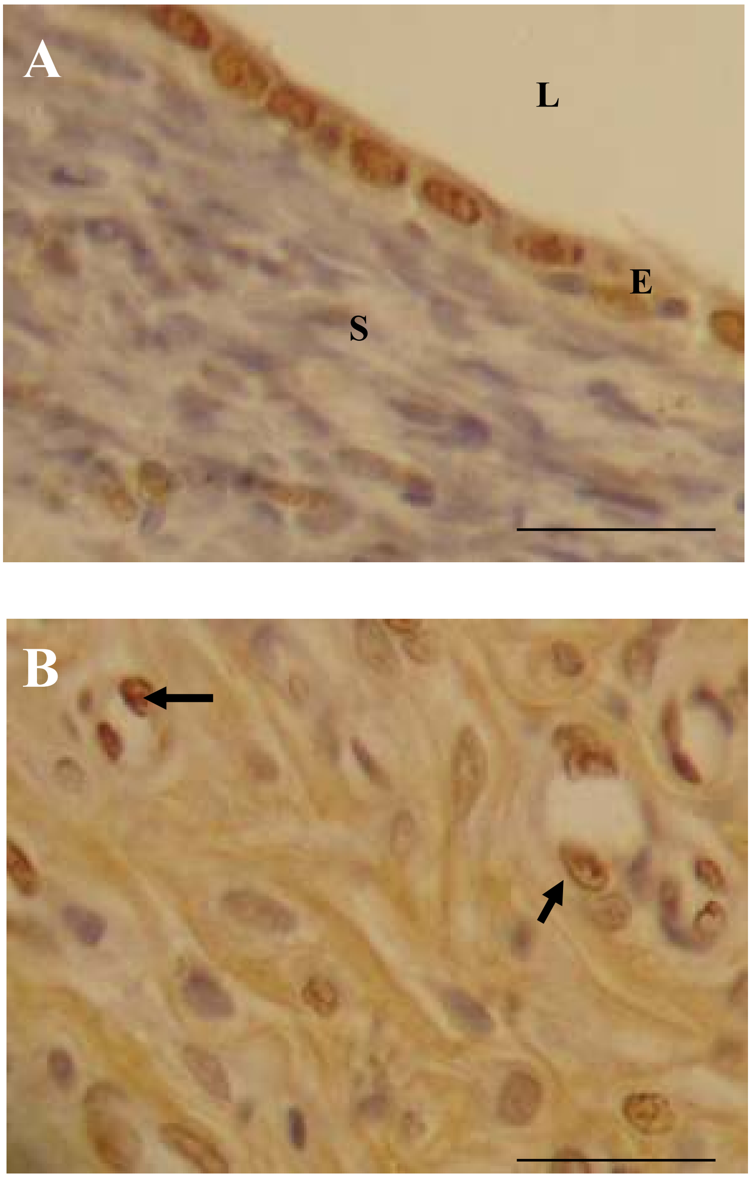


Figure 40. TUNEL assay for a β-caryophyllene-treated rat. Brown nuclei indicate the presence of apoptosis (arrow) while violet nuclei are not apoptotic. Note the presence of extensive apoptosis in luminal epithelium in A and the presence of apoptosis in endothelial cells (arrow) of some blood capillaries in B. Sections were counterstained with haematoxylin. E: luminal epithelium; L: cyst lumen; S: stroma. Scale bar=25μm in A & B.

For masoprocol-treated group, luminal epithelium was strongly positive in TUNEL assay (Figure 41). Endothelial cells of blood vessels were positive as well as few cells in the stroma, mainly fibroblasts and inflammatory cells.

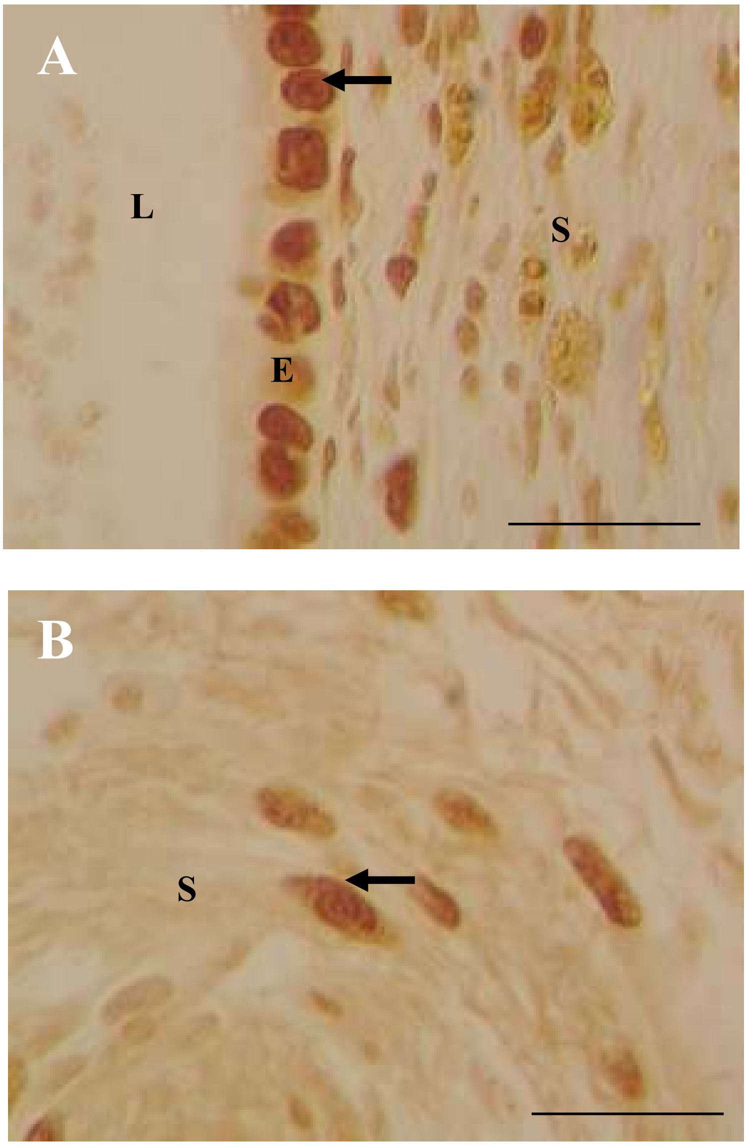


Figure 41. TUNEL assay for a masoprocol-treated rat. Luminal epithelial cells (arrow) were positive in TUNEL assay (dark brown staining) which means that they underwent apoptosis (A) as well as few cells in the stroma (B) (arrow). Note: no counter stain was used. E: luminal epithelium; L: cyst lumen; S: stroma. Scale bar=25µm in A& B.

For primaquine-treated group, luminal epithelium stained with light brown color in TUNEL assay (Figure 42). Few cells within stroma were positive too.

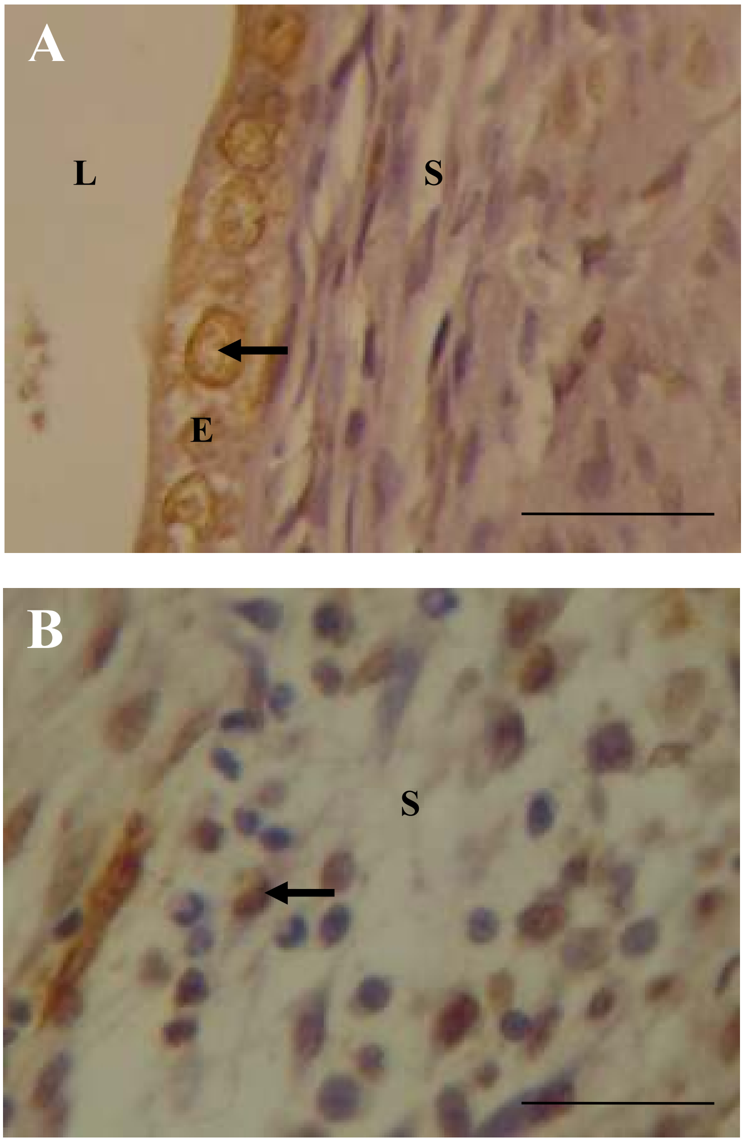


Figure 42. TUNEL assay for a primaquine-treated rat.
(A) Luminal epithelial cells (arrow) were weakly positive (faint brown staining) as well as few cells in the stroma (B) (arrow). E: luminal epithelium; L: cyst lumen; S: stroma. Scale bar=25µm in A& B.

For sunitinib-treated group, most cells in luminal epithelium were clearly positive in TUNEL assay (Figure 43). Endothelial cells of blood vessels were positive as well as some cells in stroma (Figure 44 B).

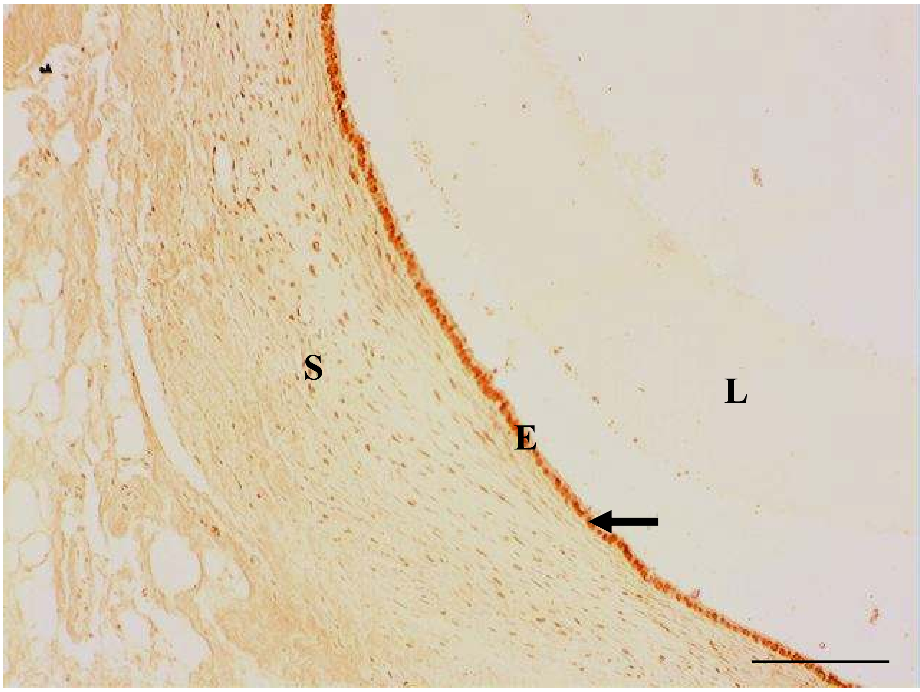


Figure 43. TUNEL assay for a sunitinib-treated rat. Luminal epithelial cells (black arrow) were positive (dark brown staining) in TUNEL assay as well as some cells in the stroma which means that they underwent apoptosis. E: luminal epithelium; L: cyst lumen; S: stroma. Scale bar=250µm.

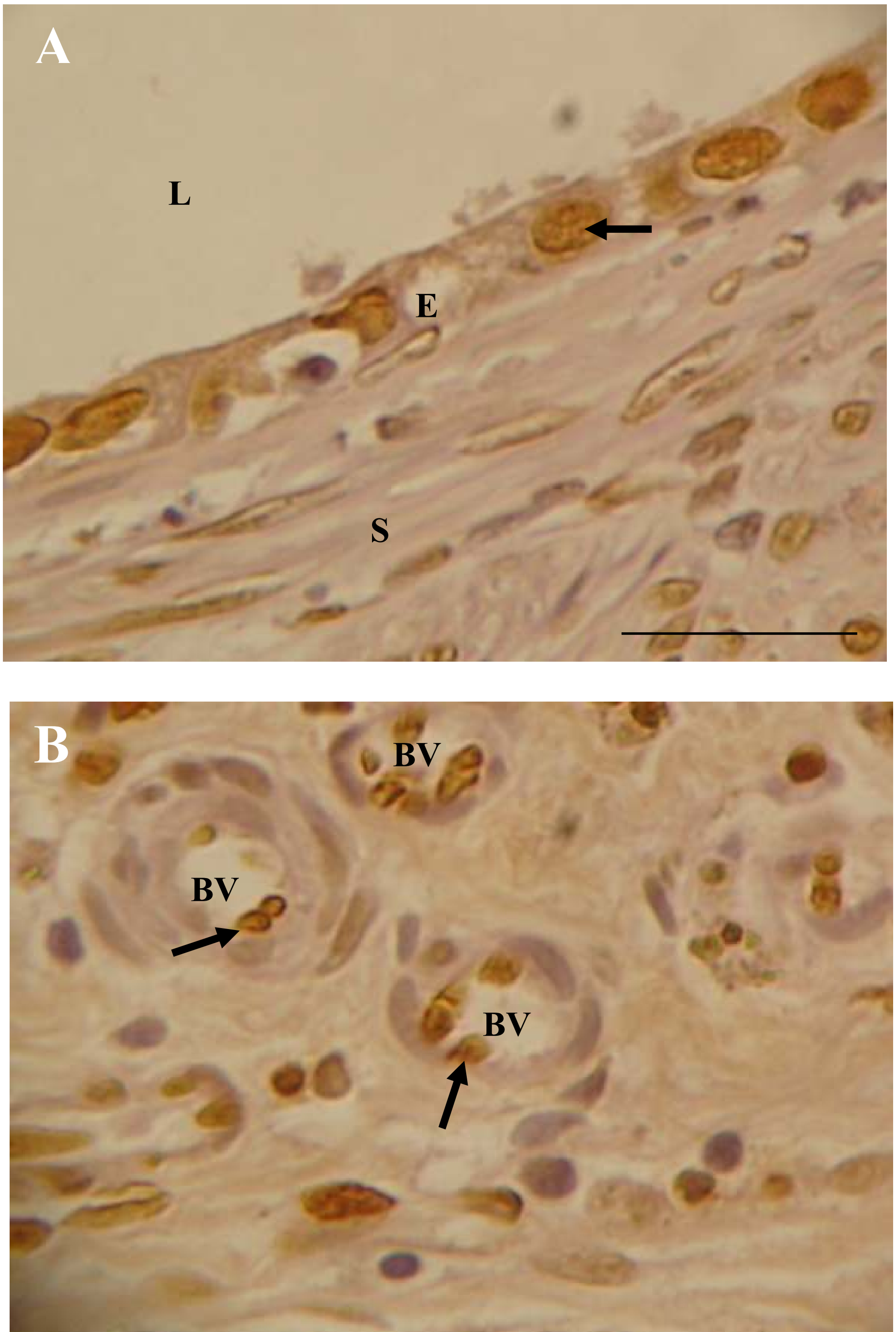


Figure 44. Positive TUNEL assay for luminal epithelial cells and endothelial cells of blood vessels of a sunitinib-treated rat. (A) Luminal epithelial cells (arrow) were positive (dark brown staining) in TUNEL assay as well as some cells in the stroma which means that they underwent apoptosis. Also, endothelial cells in B (arrow) showed positive reaction. Sections were counterstained with haematoxylin. BV: blood vessel. E: luminal epithelium; L: cyst lumen; S: stroma. Scale bar=25µm in A & B.

For theophylline-treated group, some cells in luminal epithelium were positive in TUNEL assay as well as some cells in stroma (Figure 45, 46). Some endothelial cells lining blood vessels were positive too.



Figure 45. TUNEL assay for a theophylline-treated rat.
Some luminal epithelial cells were positive in TUNEL assay as well as some cells in the stroma (white arrow) which means that they underwent apoptosis. E: luminal epithelium; L: cyst lumen; S: stroma. Scale bar= 25µm.

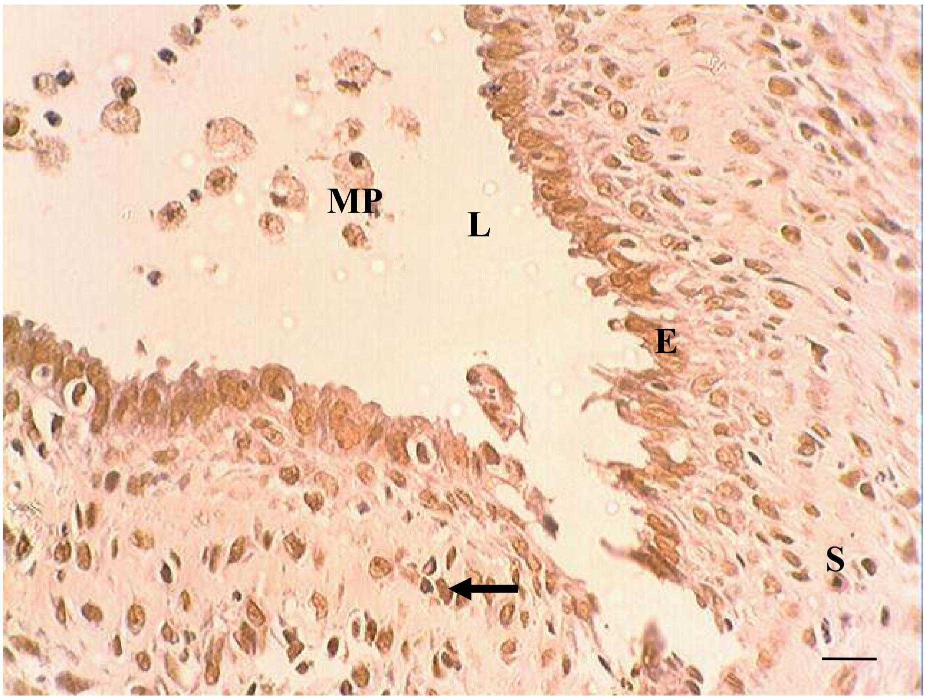


Figure 46. Positive TUNEL assay for a theophylline-treated rat.
Some luminal epithelial cells were positive (dark brown staining) in TUNEL assay as well as some cells in the stroma (arrow) which means that they underwent apoptosis. E: luminal epithelium; L: cyst lumen; MP: macrophage; S: stroma. Scale bar=100µm.

For vitamin D-treated group, TUNEL assay showed that some cells in the stroma were apoptotic including some fibroblasts and some inflammatory cells (Figure 47). Occasional apoptotic cells in luminal epithelium were seen.

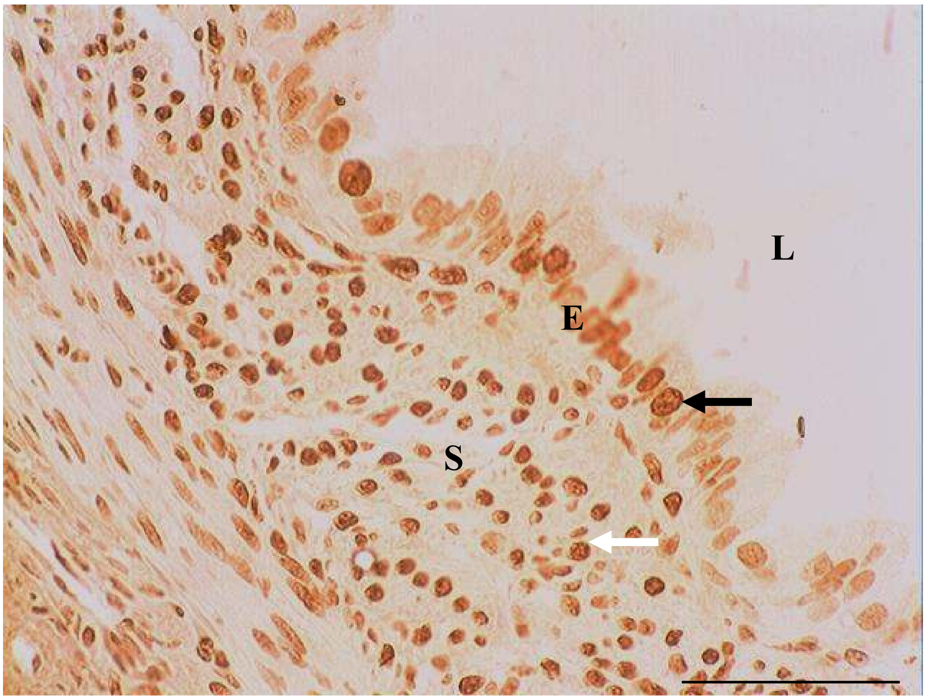


Figure 47. TUNEL assay for a vitamin D-treated rat.
(A, B) Some cells in luminal epithelium (black arrow) were positive in TUNEL assay (dark brown staining) as well as some cells in the stroma (white arrow) which means that they underwent apoptosis. E: luminal epithelium; L: cyst lumen; S: stroma. Scale bar=100µm.

4.4. Electron Microscopic Studies

Ultrastructural study of cysts revealed the presence of collagen fibers in the stroma of all studied compounds (Figure 48-52). However, their density varied. The highest density was in sunitinib-treated rats. This indicates that it has a positive effect in healing endometriotic cysts since fibrosis is usually present in healed foci of endometriosis. However, degenerating cells were also seen in sunitinib-treated specimens (Figure 51).

Mast cell granules showed variable electron densities in specimens of rats received different treatments. Granules with reduced electron density were commonly seen (Figure 52-56). In the control group, some granules were present outside the cell which indicates secretory activity of these cells. Also, small vesicles containing electron-dense material

were seen. A perigranular space that separates the perigranular membrane from the granule matrix was detected in caryophyllene (Figure 54), primaquine (Figure 55), and theophylline-treated rats (Figure 56). Most probably this leads to the extrusion of granule contents by the fusion of their membrane with the cell membrane.

Eosinophils were commonly observed in electron microscopic images (Figure 57-62). Eosinophils of the control and caryophyllene-treated groups revealed the presence of granules with dark crystals and granules with light crystals (Figure 57, 58). The chromatin of an eosinophil in a caryophyllene-treated rat was condensed and the whole cell shrinked (Figure 59). This indicates that it underwent apoptotic cell death. Also eosinophils that underwent cytolysis were seen in caryophyllene-treated group (Figure 60). This cell exhibited loss of integrity of plasma membrane and spilling of its contents into the surrounding tissue. Release of intact or disrupted granules into the interstitium is likely to have toxic effects on the surrounding cells.

Electron micrographs of eosinophils from endometriotic tissue of theophylline and vitamin D-treated groups showed only granules with dark crystals (Figure 61, 62). The core of eosinophil granules is a crystal containing major basic protein. Secretion of this protein contributes to the immunopathology of various diseases. In some granules of mast cells of vitamin D-treated rat, two crystals were observed (Figure 62).

In addition to mast cells and eosinophils, other types of inflammatory cells were detected including plasma cells and macrophages (Figure 63). Macrophages were commonly seen in the stroma. Endometrial cells express the chemotactic factor (MCP-1) responsible for the grouping and activation of macrophages.

A longitudinal section in a small blood vessel of a control rat (Figure 64) and in a suntinib-treated rat (Figure 66) does not indicate a clear abnormality in their structure. Also, Figure 65 shows a cross-section in an apparently normal small blood vessel in theophyllene treated group. This figure can be considered as a representative for the control group. In this figure two endothelial cells appear resting on a basement membrane.Erythrocytes were present in the lumen of this blood vessel. Furthermore, a pericyte is seen in the figure.

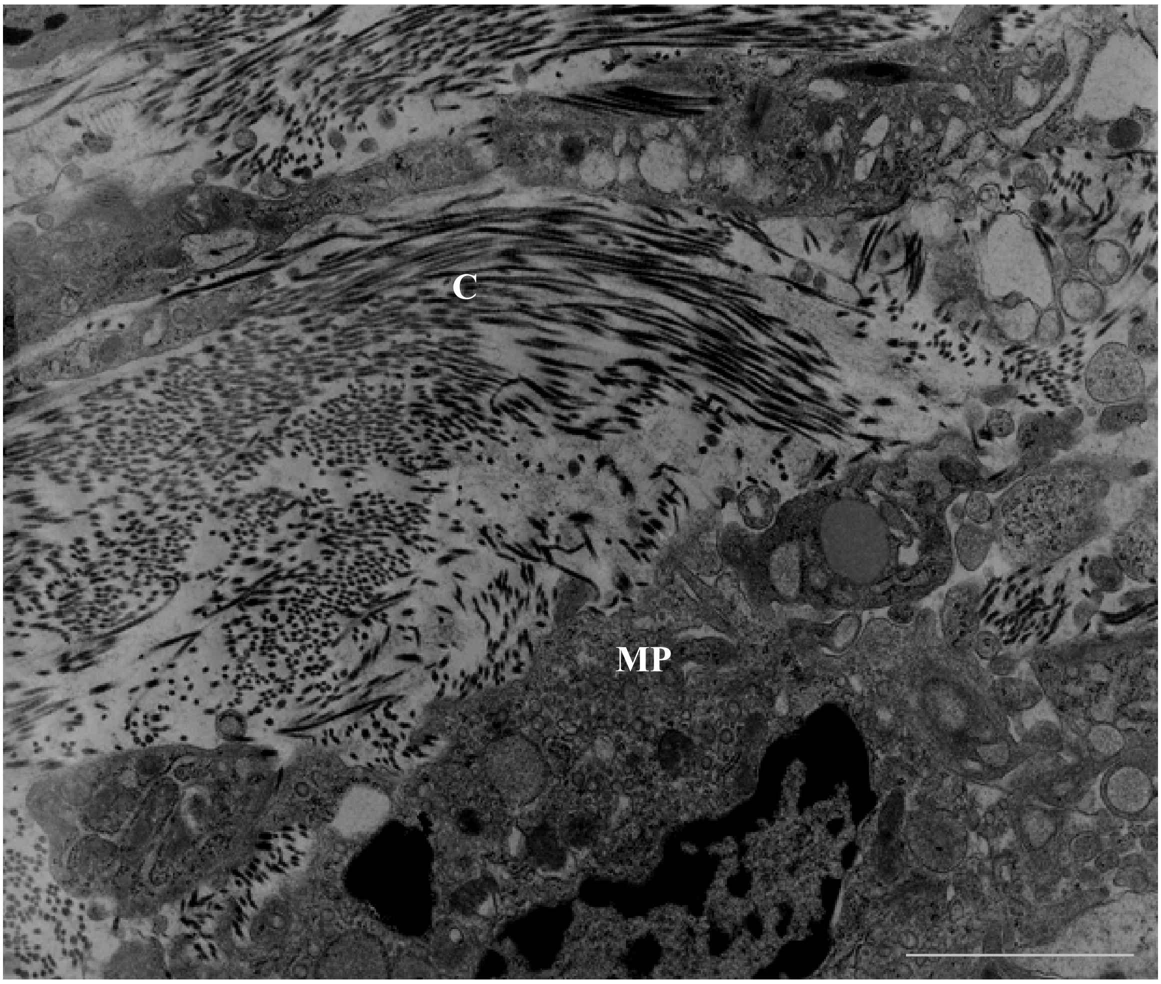


Figure 48. Fibrosis in a caryophyllene-treated rat. This image shows collagen fibers and a part of a macrophage. Fibrosis is usually present in healed foci of endometriosis. C: collagen fibers; MP: macrophage. Scale bar=2.08μm.

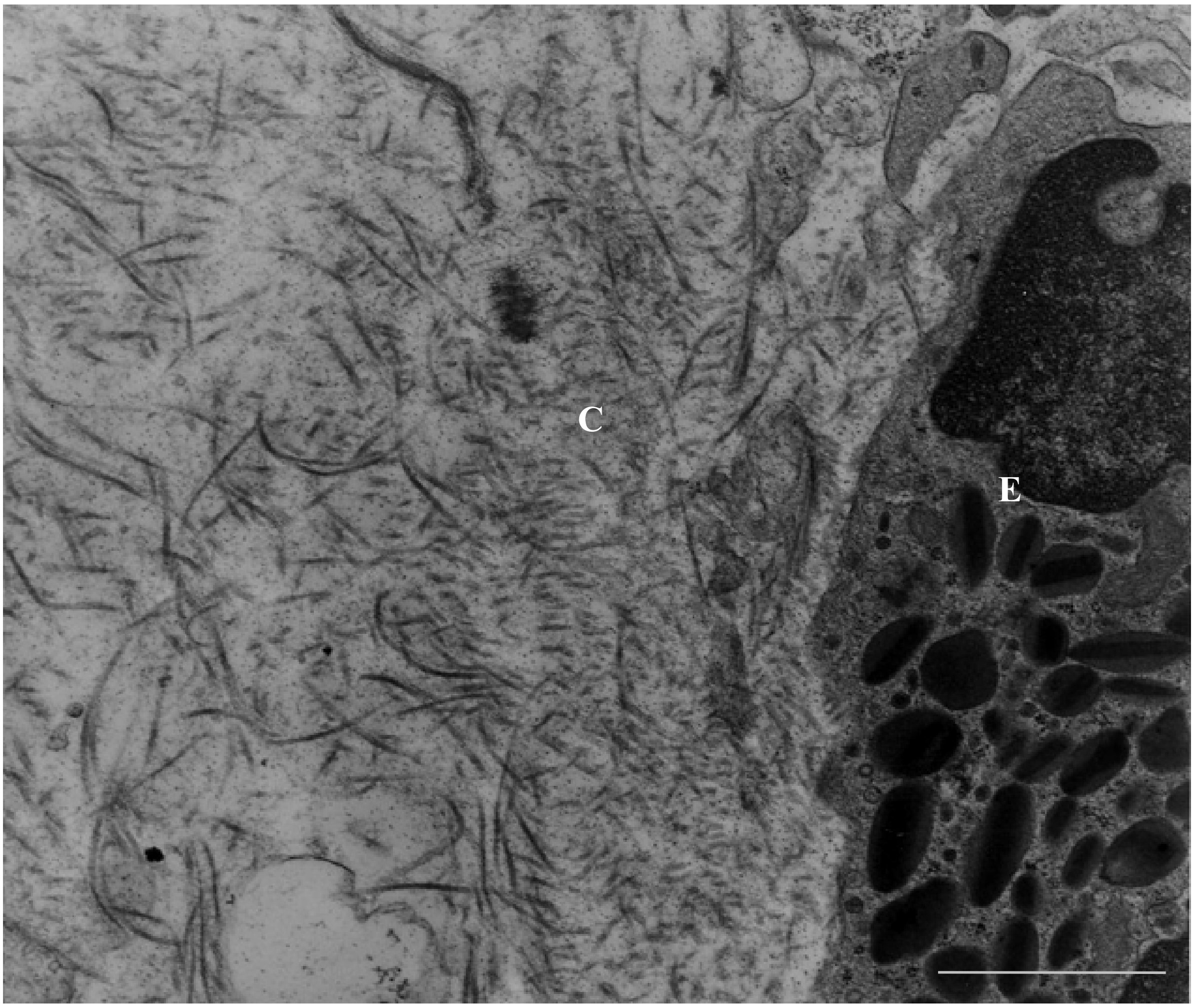


Figure 49. Fibrosis in a primaquine-treated rat. In this electron micrograph an eosinophil is seen with newly formed collagen in extracellular matrix. C: collagen fibers; E: eosinophil. Scale bar=1.3μm.

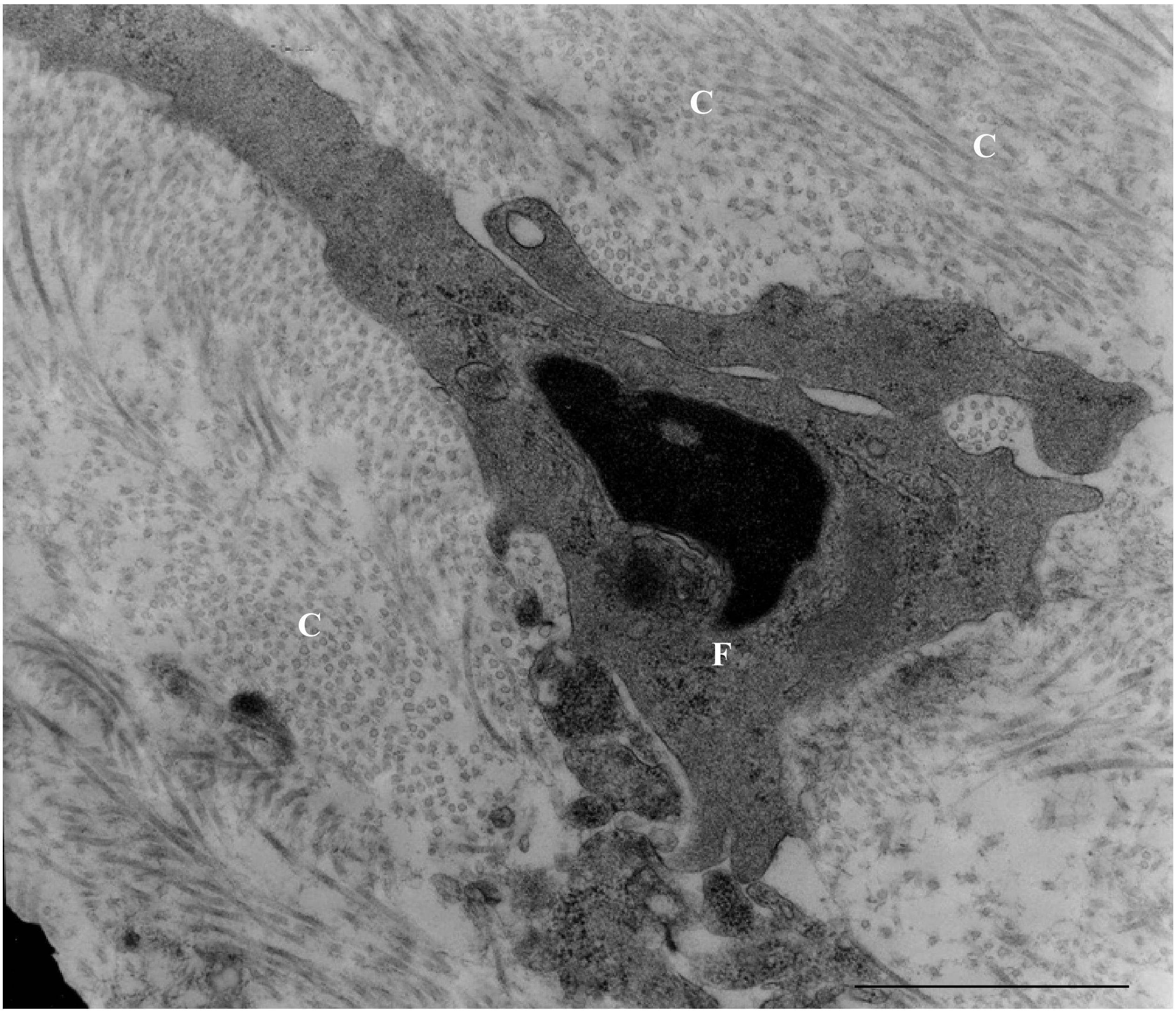


Figure 50. Fibrosis in a sunitinib-treated rat. In this electron micrograph a fibroblast is seen in a matrix containing collagen fibers. This indicates that sunitinib has a positive effect in healing endometriotic cysts. C: collagen fibers; F: fibroblast. Scale bar=1.25µm.

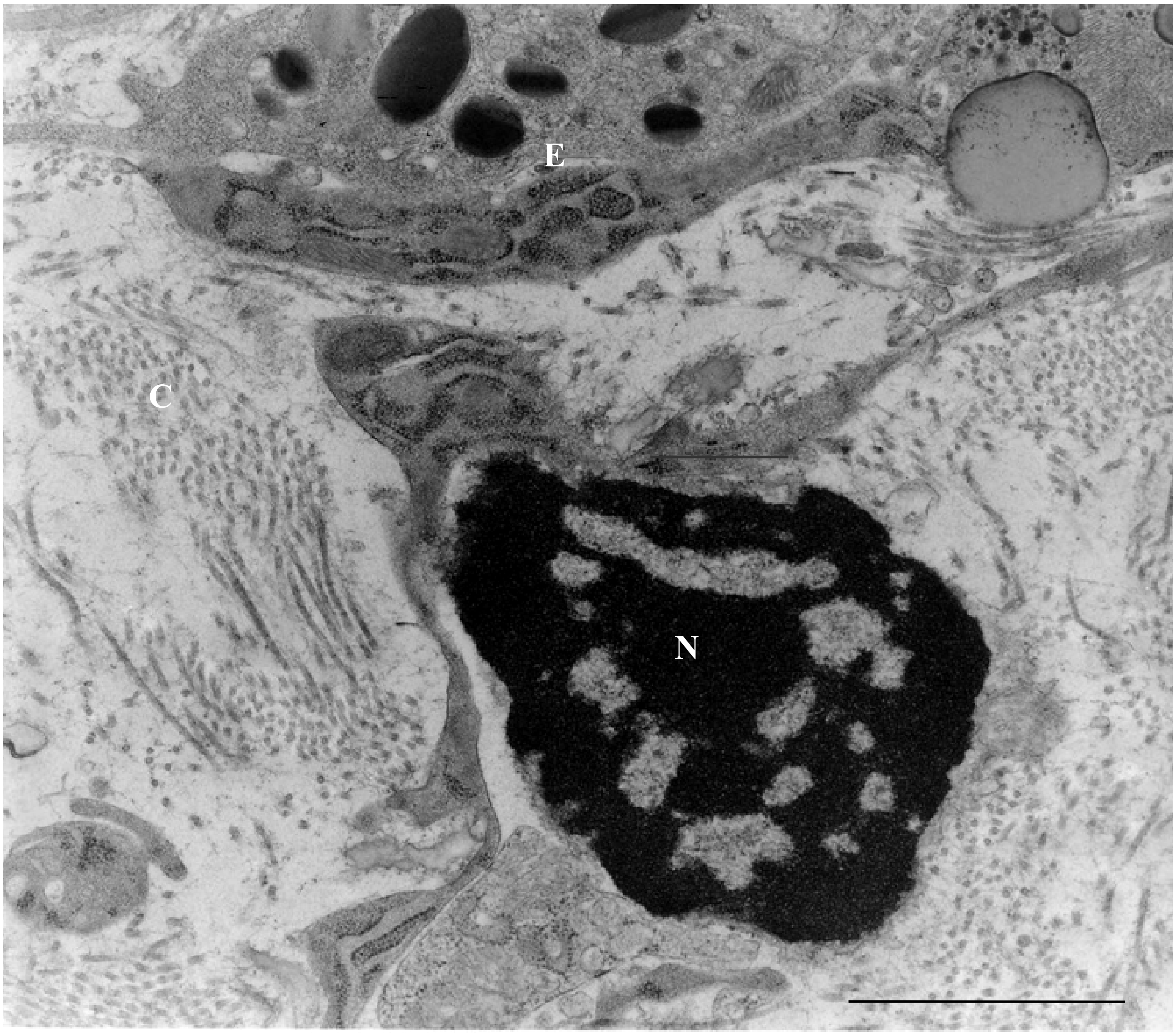


Figure 51. Electron micrograph of a cell that underwent necrosis in a sunitinib-treated rat. In this electron micrograph collagen fibers are seen in extracellular matrix. An eosinophil appears on top. Another cell with a ruptured cell membrane is also seen. This indicates that sunitinib produces degenerative changes in certain cells in the stroma. C: collagen fibers; E: eosinophil. N: necrotic cell. Scale bar=1.6μm.

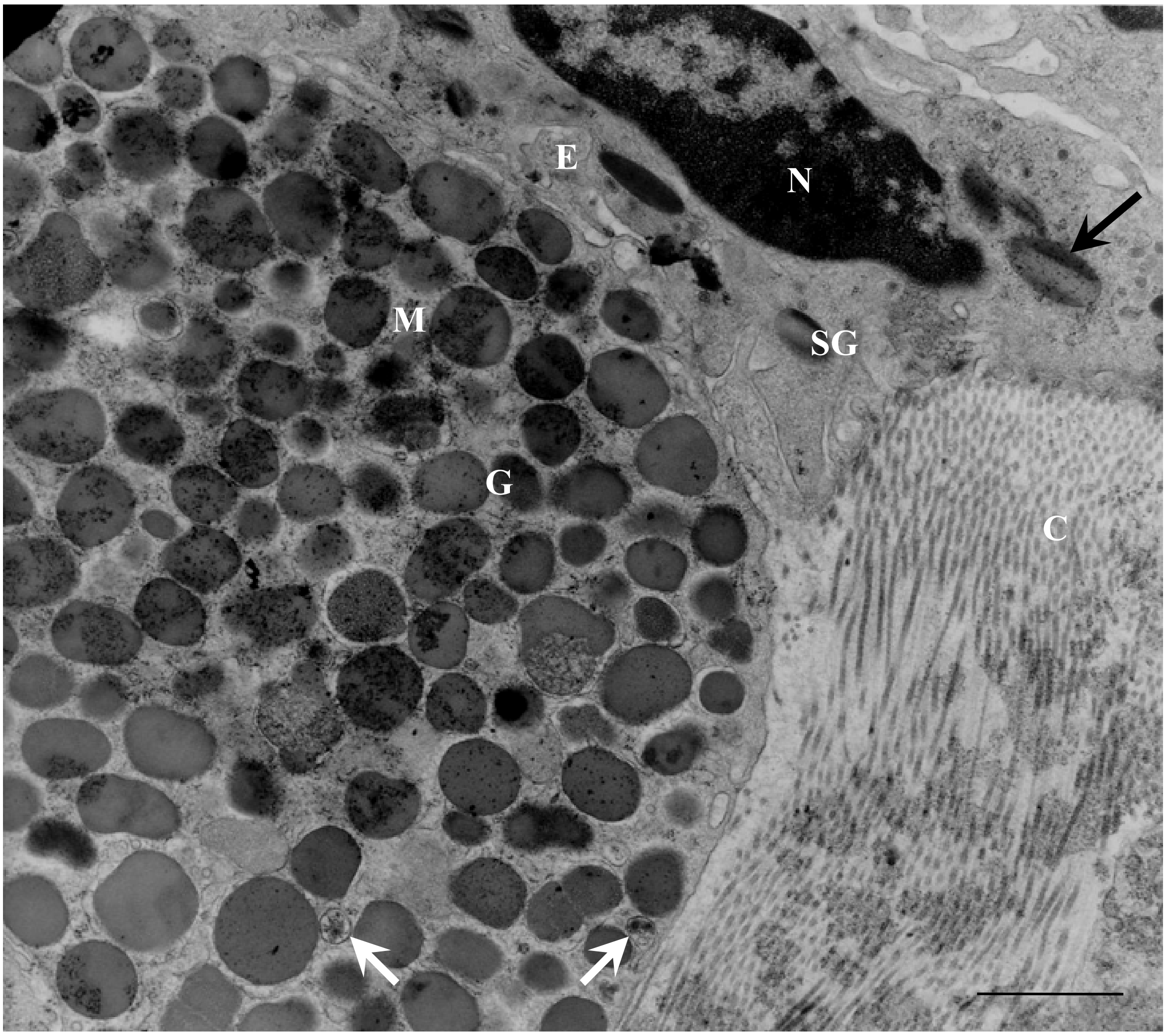


Figure 52. Electron micrograph of a mast cell of a control rat.
This image shows a mast cell and an eosinophil. Mast cell granules show variable electron densities. Small vesicles containing electron-dense material are seen (white arrows). Some granules in the eosinophil contain light crystals (black arrow). Collagen fibers are present in extracellular matrix. C: collagen fibers; E: eosinophil; G: granule; M: mast cells; N: nucleus; SG: specific granules. Scale bar=1.05µm.

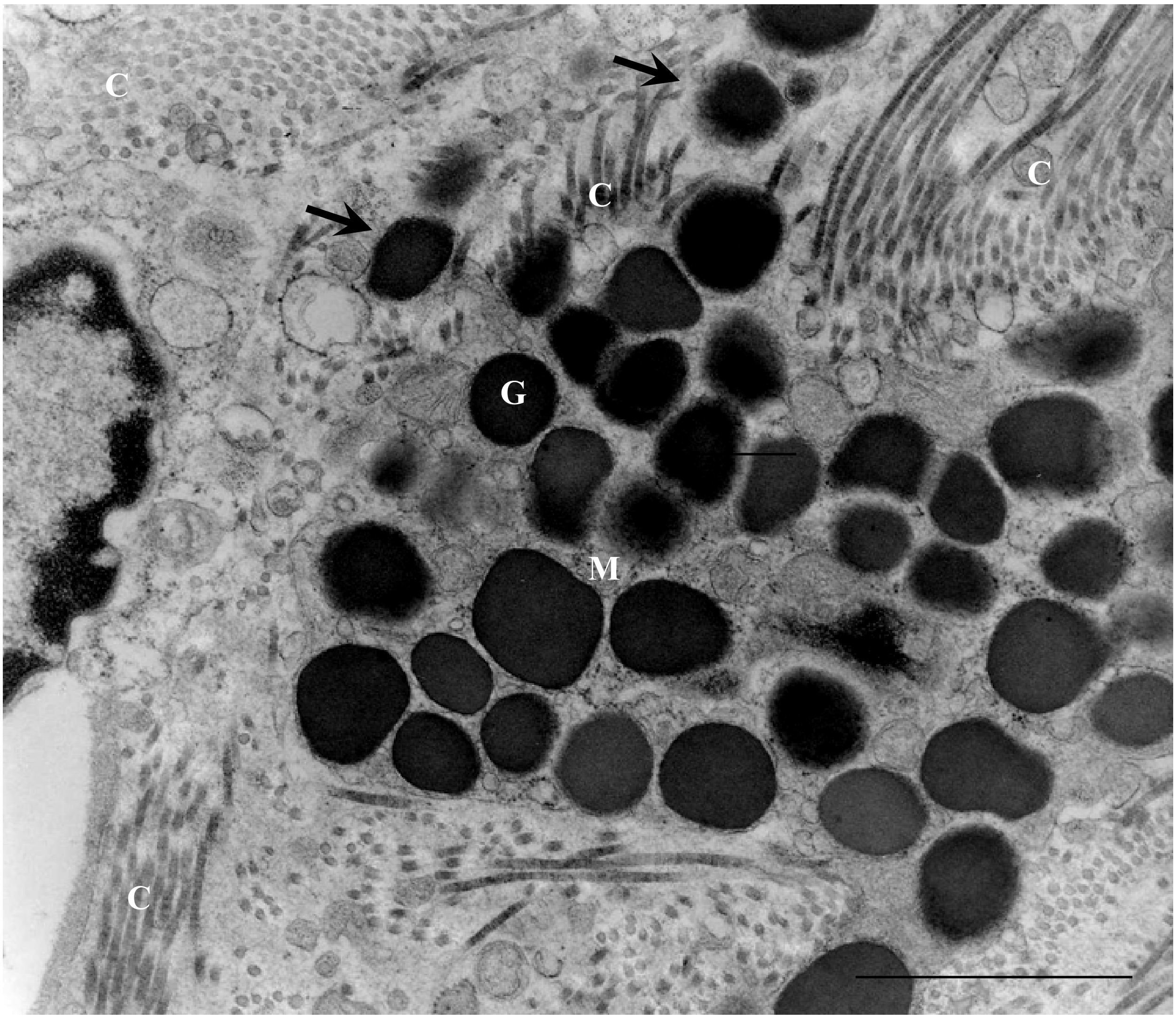


Figure 53. Electron micrograph of a degranulated mast cell of a control rat. In this electron micrograph a part of a mast cell is seen with granules showing variable electron densities. Note the presence of some granules outside the cell (arrows) which indicates secretory activity of these cells. C: collagen fibers; G: granule; M: mast cells. Scale bar=1.25μm.

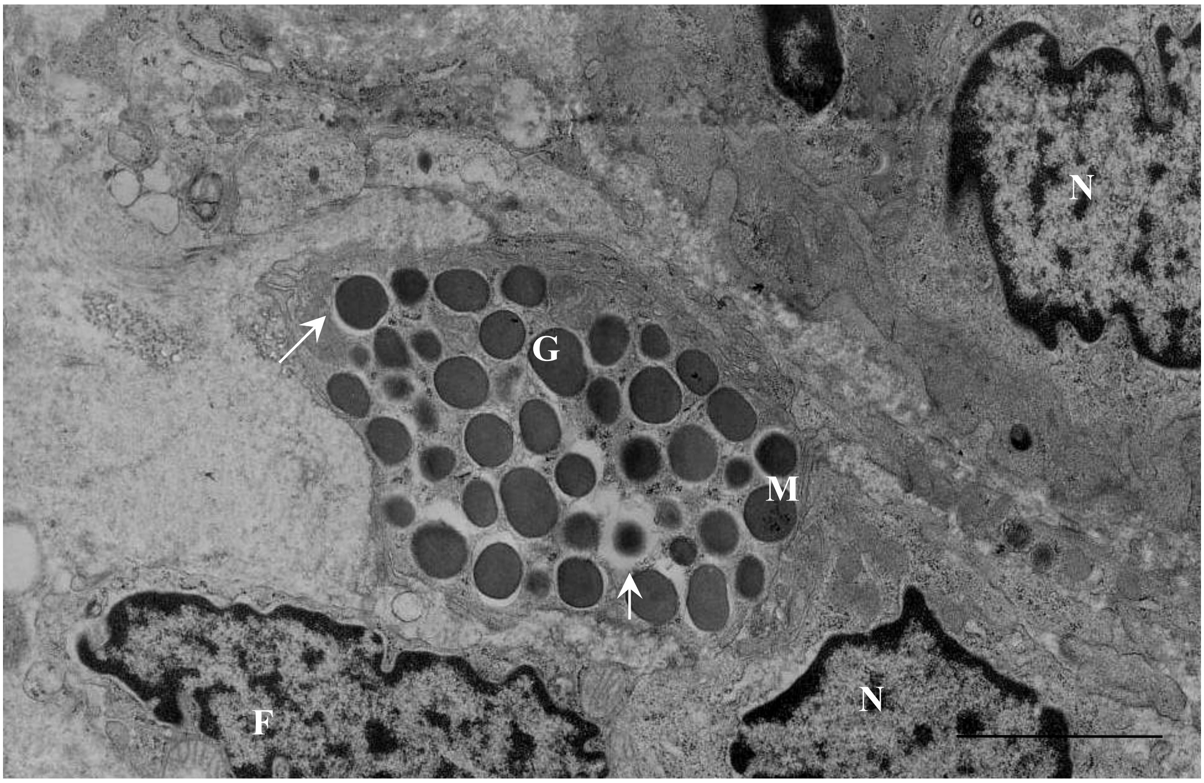


Figure 54. Electron micrograph of a mast cell of a caryophyllene-treated rat. This image shows a mast cell containing granules with variable electron densities. A perigranular space separates the perigranular membrane from the granule matrix (arrow) which will be followed with fusion with the cell membrane. F: fibroblast; G: granule; M: mast cells; N: nucleus. Scale bar=2.2µm.

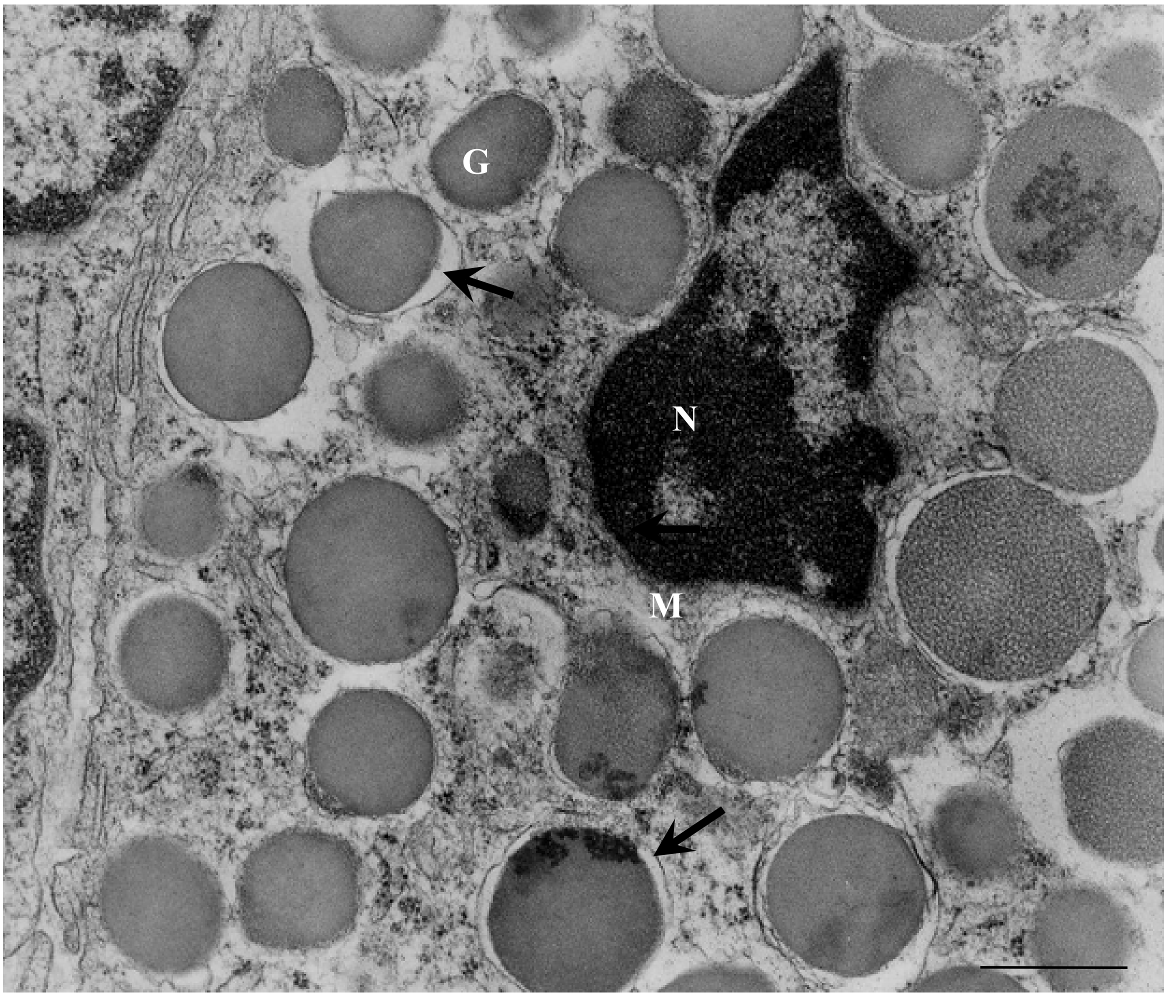


Figure 55. Electron micrograph of a mast cell of a primaquine-treated rat. The perigranular membrane is separated from the granule matrix by a perigranular space (arrow). Fusion with the cell membrane will follow which leads to extrusion of granule contents. G: granule; M: mast cells; N: nucleus. Scale bar=0.53µm.

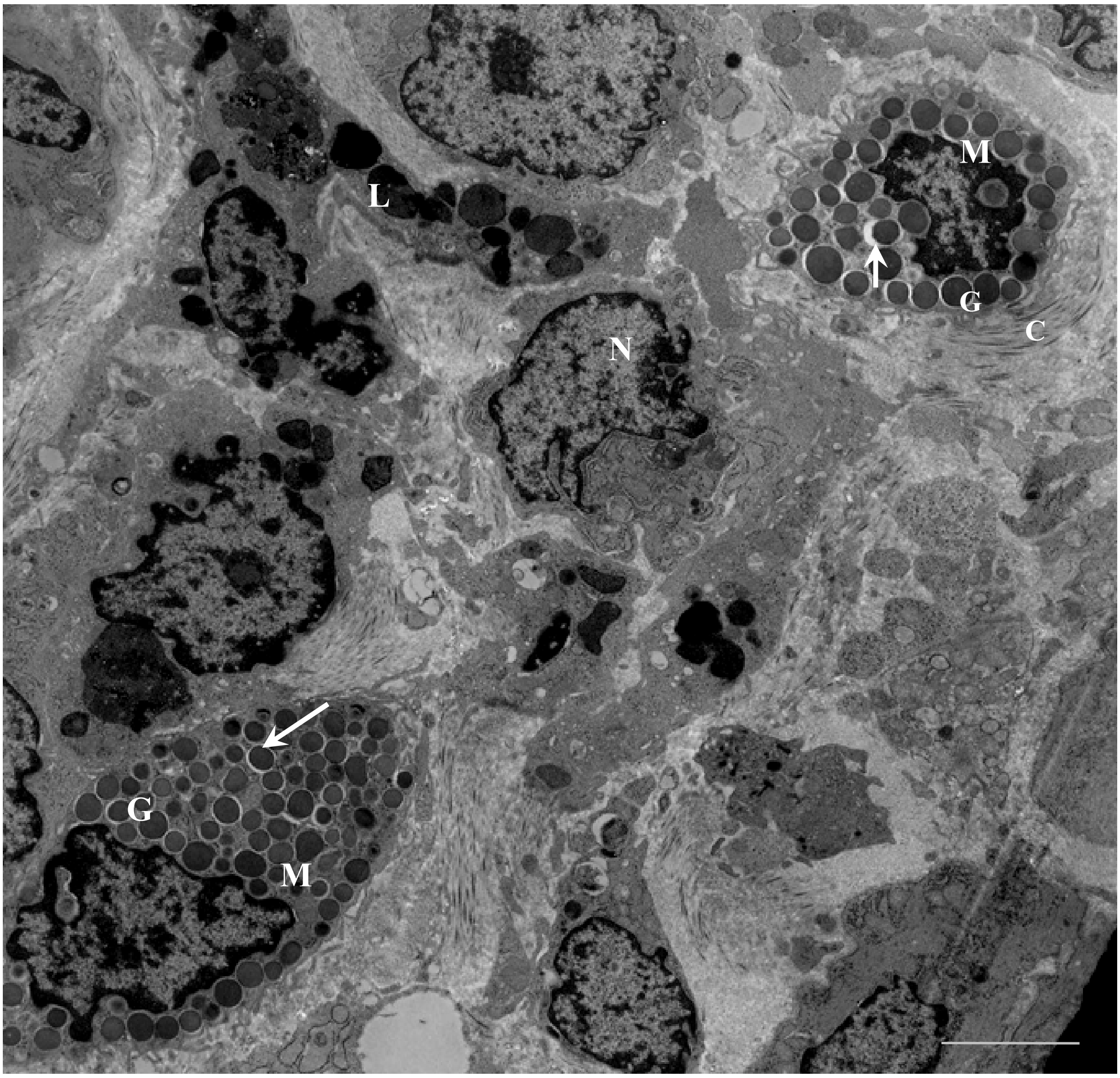


Figure 56. Electron micrograph of a mast cell of a theophylline-treated rat. In this electron micrograph two mast cells are seen in which granules have variable electron densities. A perigranular space separates the perigranular membrane from the granule matrix (arrows) followed by externalization of granule membranes. C: collagen fibers; G: granule; L: lysosomes; M: mast cells; N: nucleus. Scale bar=3.8µm.

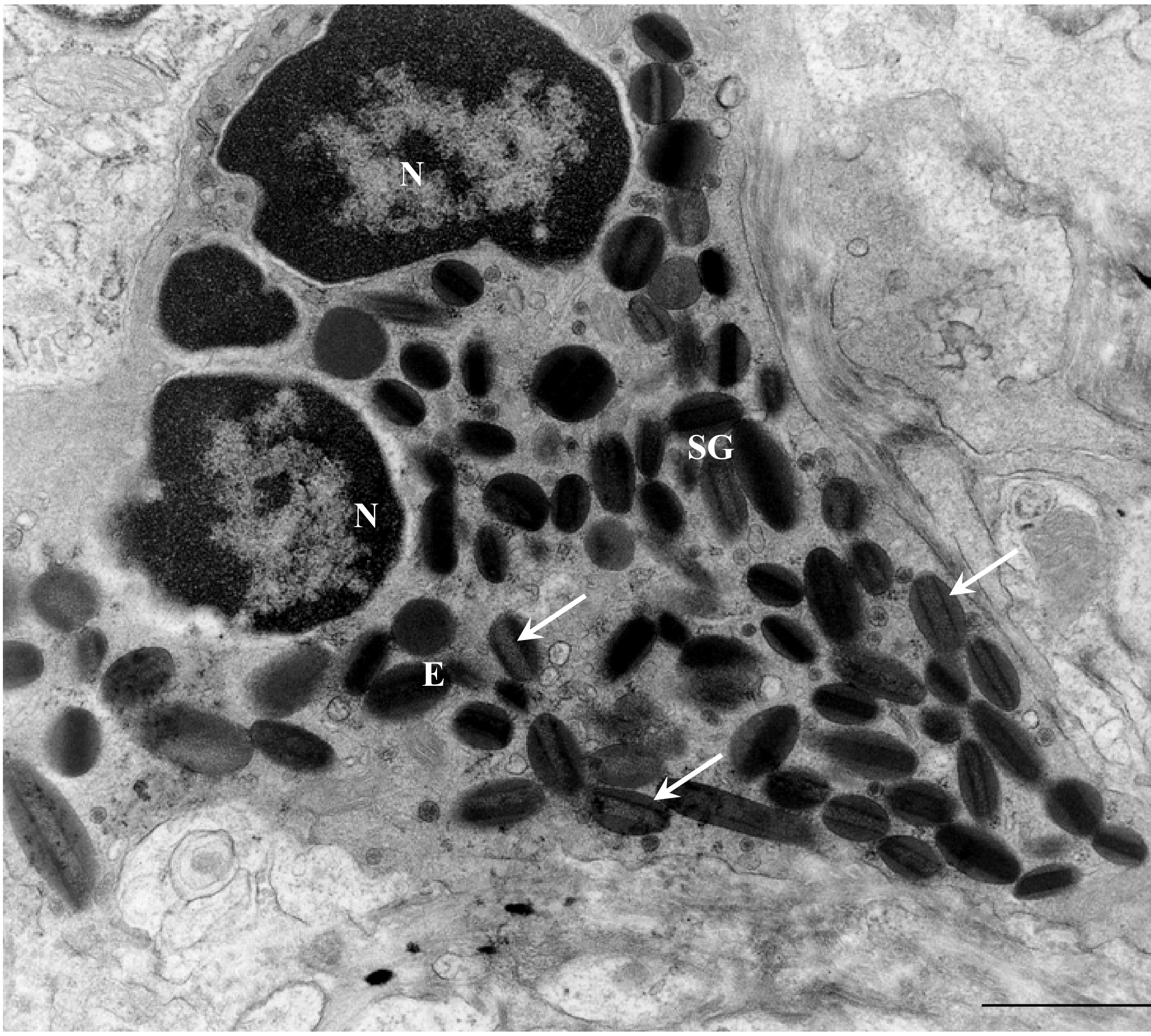


Figure 57. Electron micrograph of an eosinophil of a contol rat. In this image granules with dark crystals and granules with light crystals (arrow) are seen. E: eosinophil; N: nucleus; SG: specific granules. Scale bar=1.08µm.

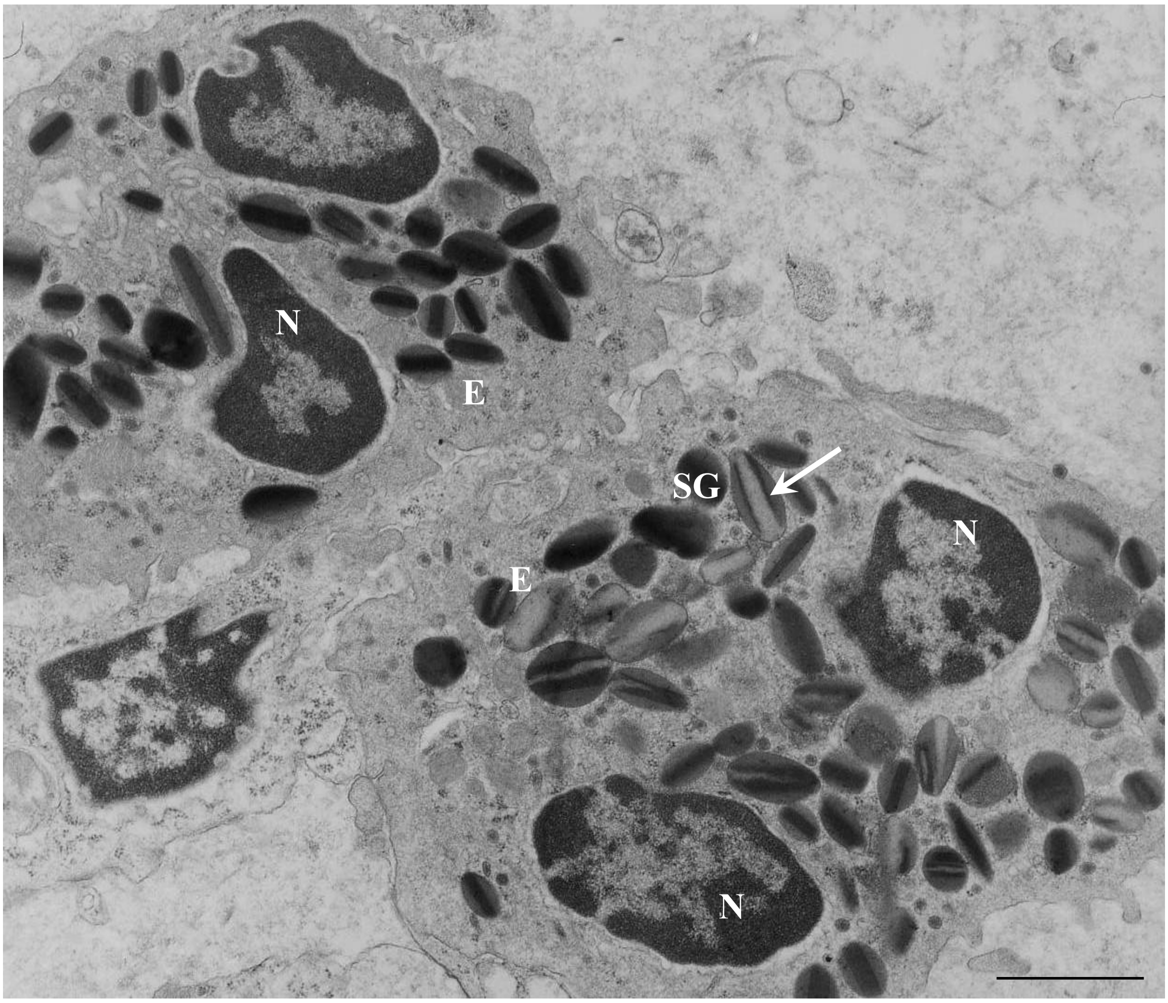


Figure 58. Electron micrograph of an eosinophil of a caryophyllene-treated rat. In this image granules with dark crystals and granules with light crystals (arrow) are seen. The core of eosinophil granules is a crystal containing major basic protein. Secretion of this protein contributes to the immunopathology of various diseases. E: eosinophil; N: nucleus; SG: specific granules. Scale bar=1.06μm.

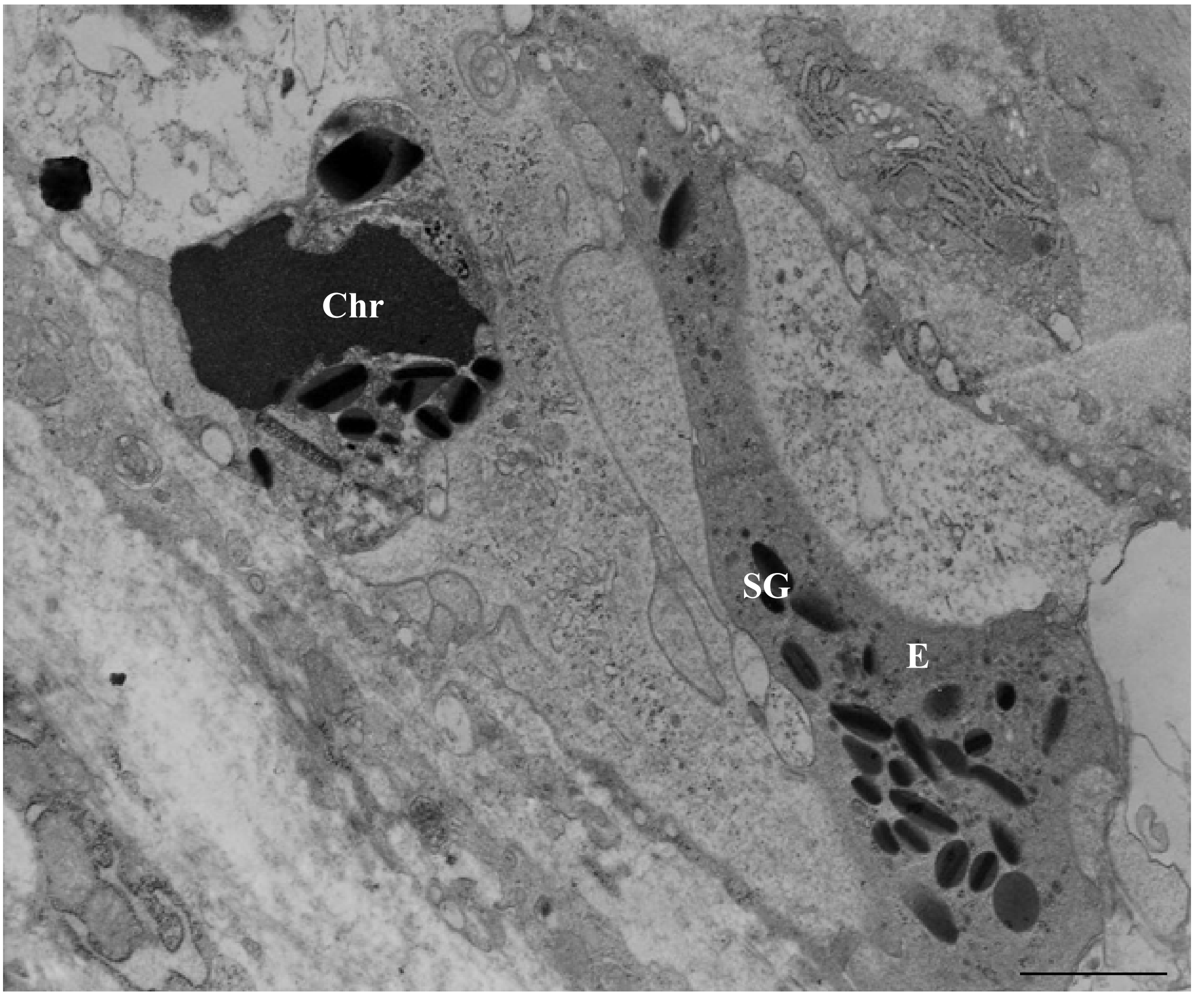


Figure 59. Electron micrograph of an abnormal eosinophil of a caryophyllene-treated rat. In this image, two eosinophils are seen. The upper one shows cell shrinkage and chromatin condensation. Mostly, it undergoes apoptotic cell death. The other eosinophils appear normal. Chr: condensed chromatin; E: eosinophil; SG: specific granules. Scale bar=1.3μm.

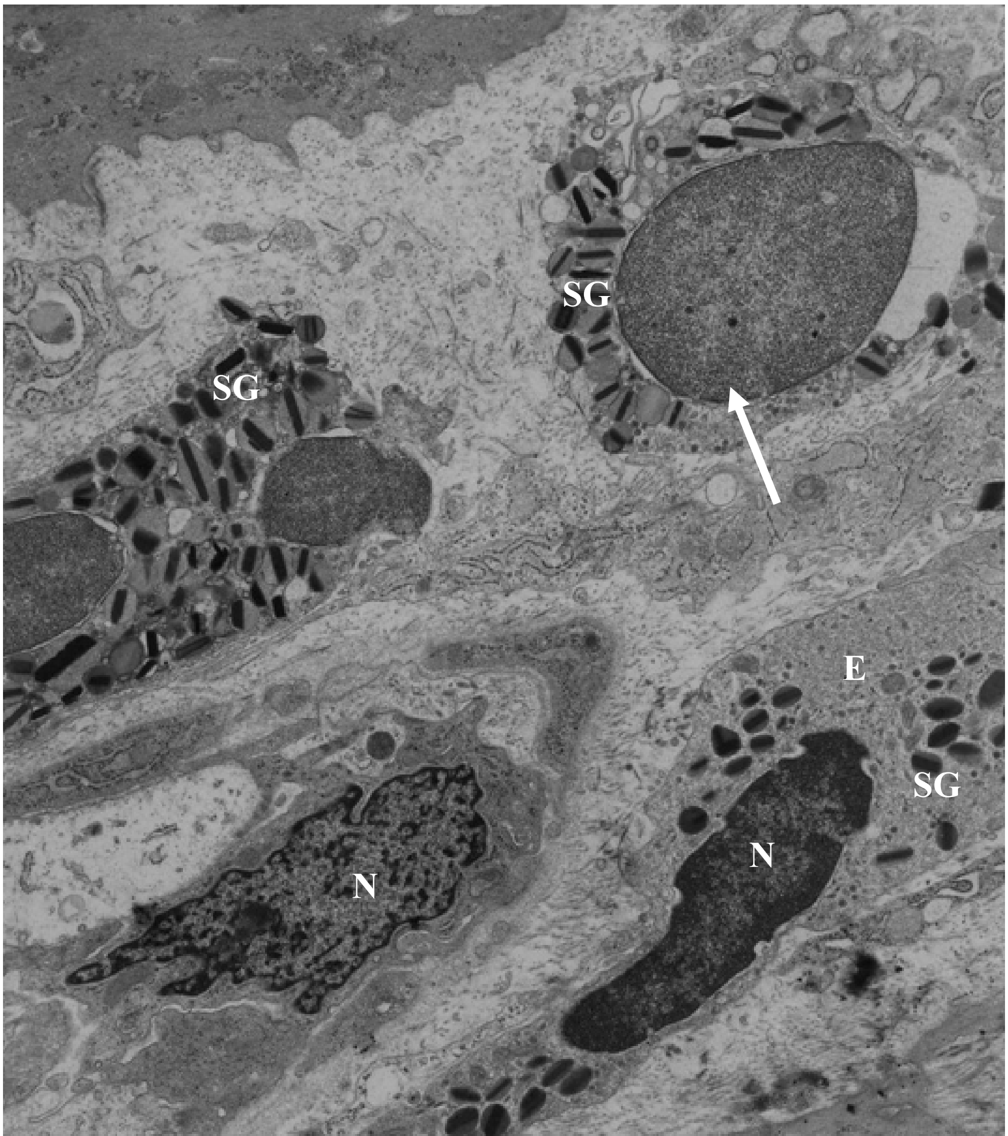


Figure 60. Electron micrograph of an eosinophil that underwent cytolysis of a caryophyllene-treated rat. In this image, three eosinophils are seen. The upper one underwent cytolysis (arrow). This cell exhibited loss of integrity of plasma membrane and spilling of its contents into the surrounding tissue. Release of intact or disrupted granules into the interstitium is likely to have toxic effects on the surrounding cells. E: eosinophil; N: nucleus; SG: specific granules. Scale bar=2.1µm.

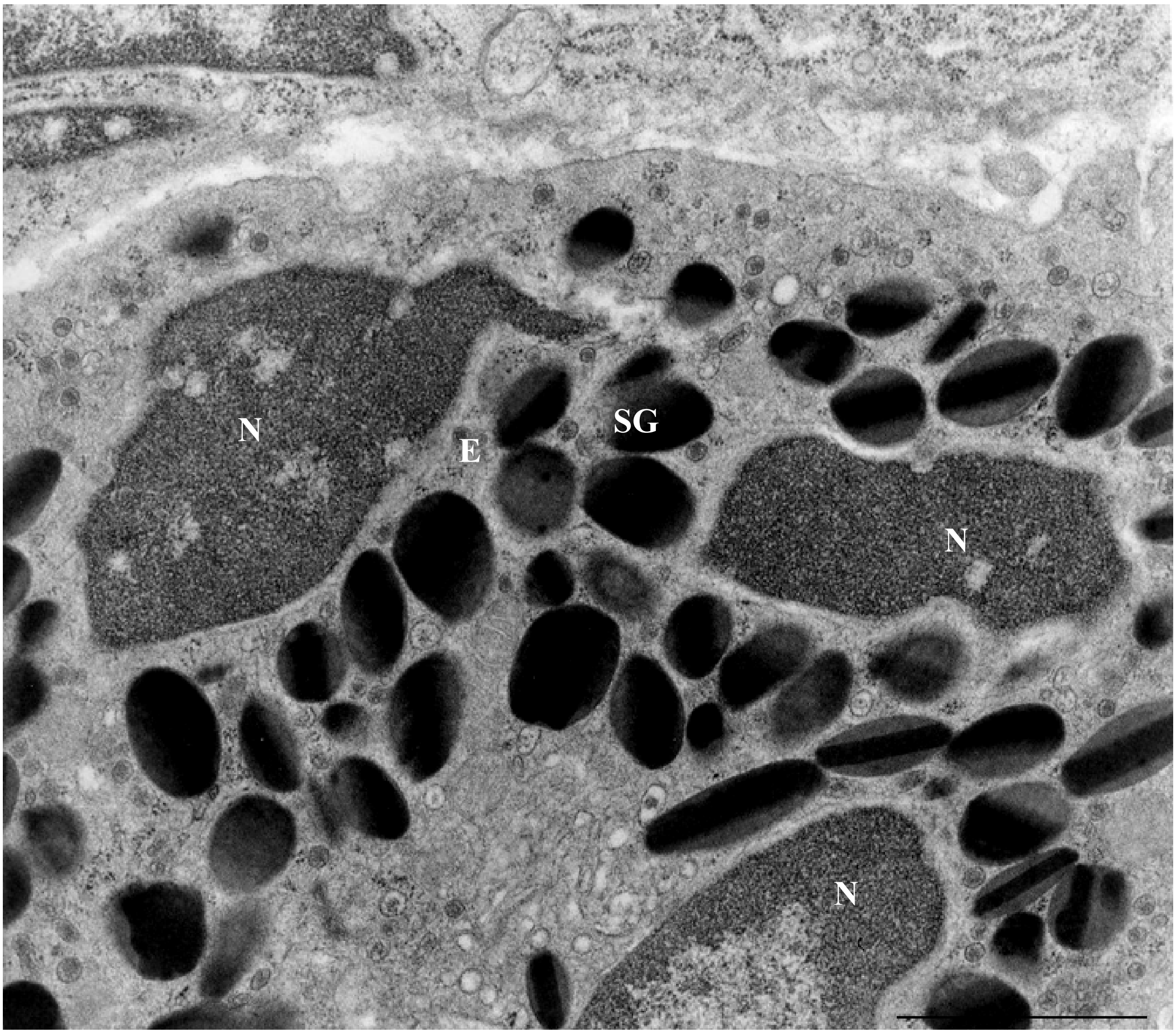


Figure 61. Electron micrograph of an eosinophil of a theophylline-treated rat. In this figure most granules have dark crystals which may indicate that theophylline inhibits eosinophil degranulation. E: eosinophil; N: nucleus; SG: specific granules. Scale bar=1.1µm.

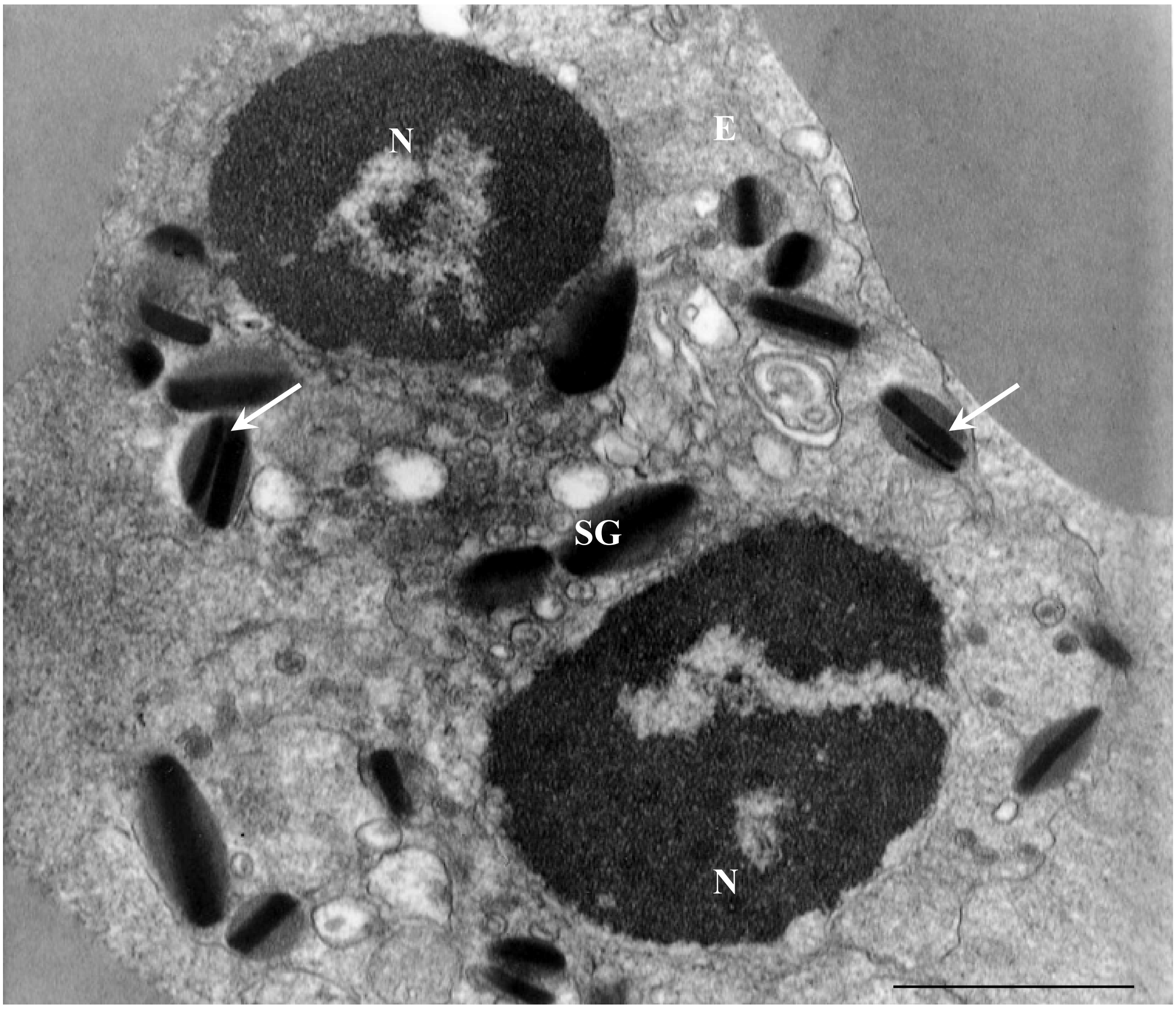


Figure 62. Electron micrograph of an eosinophil of a vitamin D-treated rat. In this figure, most granules have one or two (arrows) dark crystals which may indicate that vitamin D inhibits the release of contents from eosinophil granules. E: eosinophil; N: nucleus; SG: specific granules. Scale bar=1.1µm.

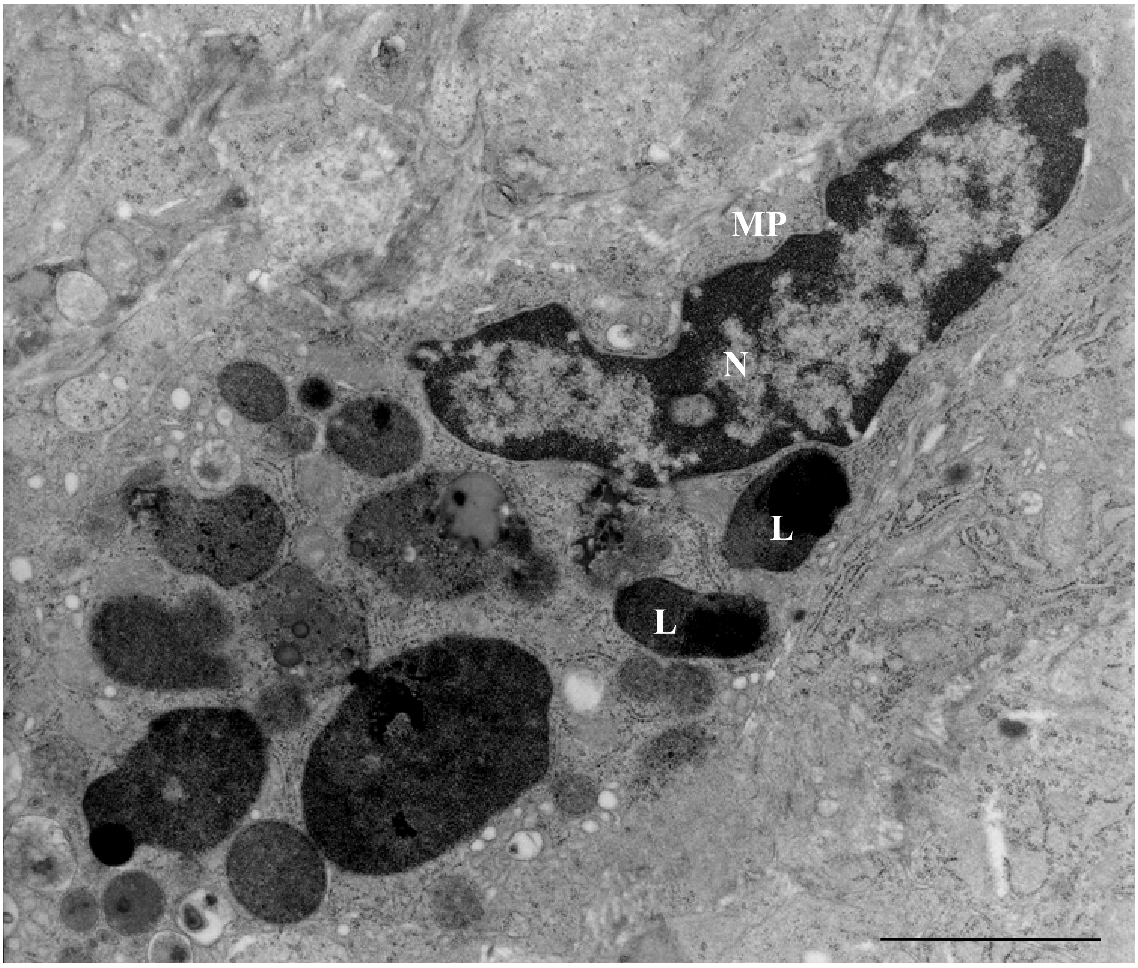


Figure 63. Electron micrograph of a macrophage of a theophylline-treated rat. Macrophages were commomly seen in the stroma. Endometrial cells express the chemotactic factor (MCP-1) responsible for the grouping and activation of macrophages. L: lysosome; MP: macrophage; N: nucleus; Scale bar=3.1µm.

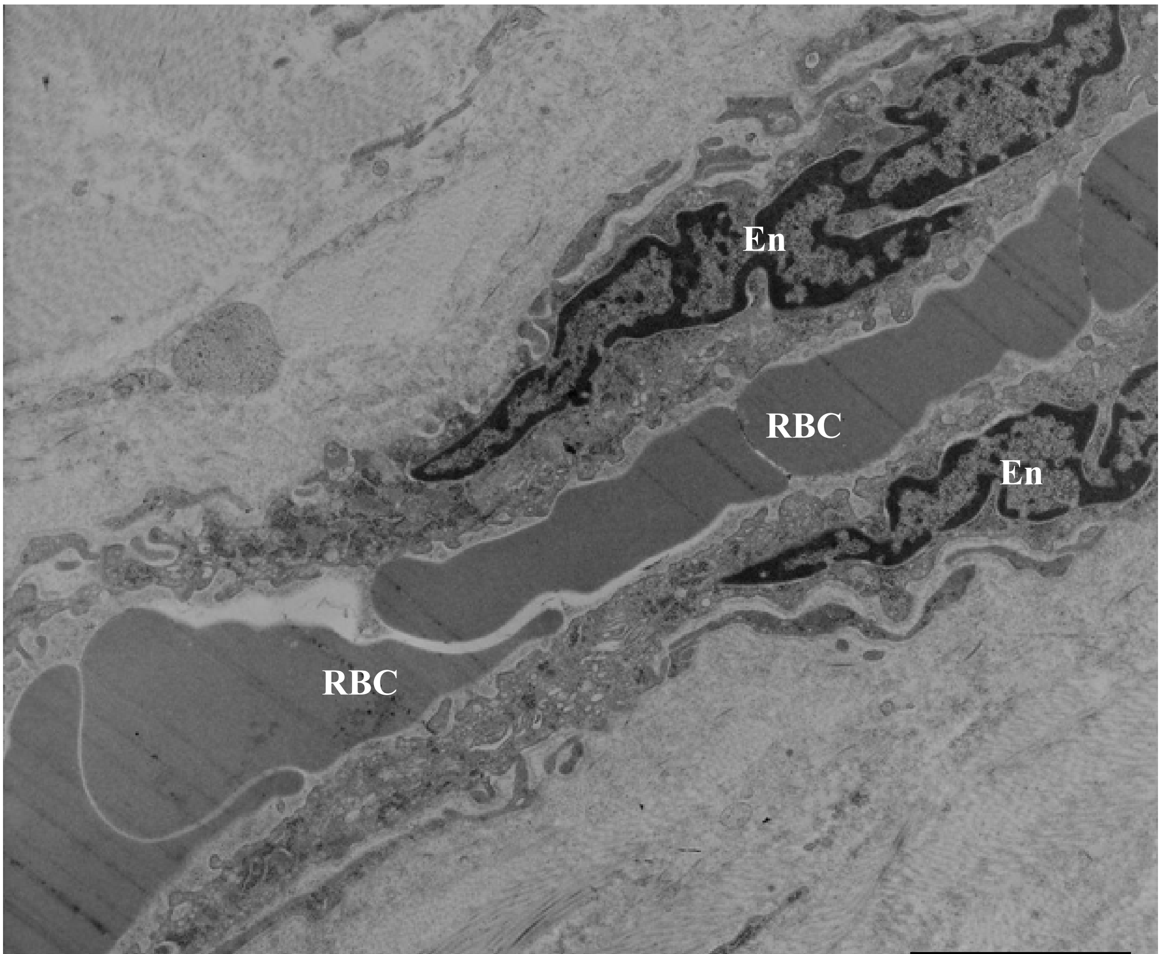


Figure 64. Electron micrograph of a capillary of a control-rat. En: endothelial cell; RBC: red blood cell. Scale bar=4.0µm.

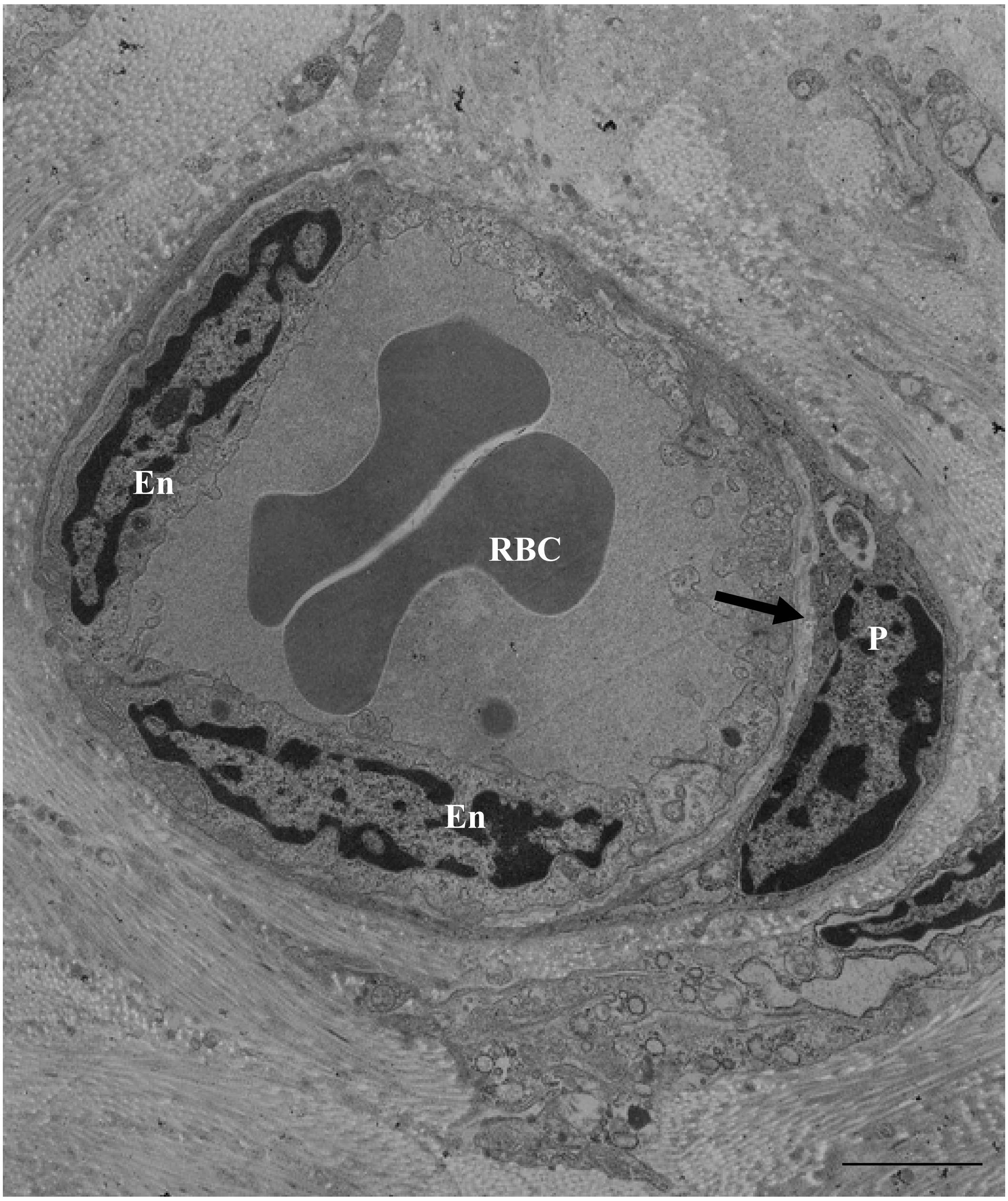


Figure 65. Electron micrograph of a small blood vessel in a theophylline-treated rat. In this figure, two endothelial cells appear resting on a basement membrane (black arrow). Two erythrocytes are present in the lumen of this blood vessel. Also, a pericyte is seen. Perictes keep the integrity of blood vessels. Also, they have a contractile function and participate in the repair process. En: endothelial cell; P: pericyte; RBC: red blood cell. Scale bar=2.0µm.

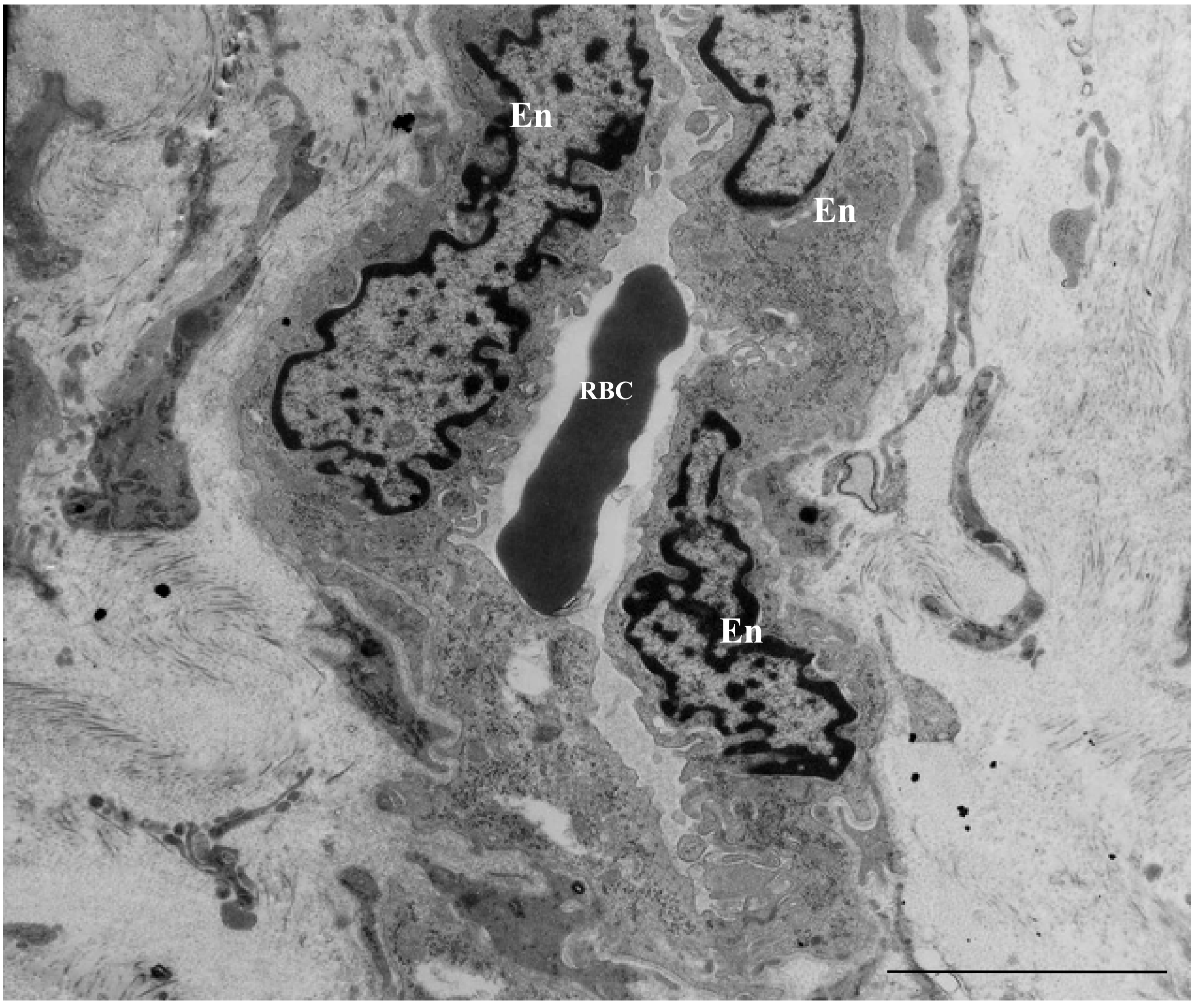


Figure 66. Electron micrograph of a small blood vessel of a sunitinib-treated rat. In this figure, an erythrocyte is present in the lumen of a small blood vessel. En: endothelial cell; RBC: red blood cell. Scale bar=4.0µm.

4.5. Antiangiogenic Effect of β-Caryophyllene (Aortic Ring Assay)

Results of aortic ring assay are shown in Table 8 and Figure 67. Neither 1 µM nor 10 µM were able to inhibit the growth of capillaries. However a 51% inhibition was achieved using 100 µM concentration.

Table 8. Distance of blood vessels from aortic ring

Concentration	Control (DMSO)	1 µM	10 µM	100 µM
Average (mm)	26.7	25.26	31.80	13.08 *
Standard Deviation	0.47	2.96	3.48	4.48
% Inhibition		5.4	-19.1	51.0

- Statistically significant difference $p<0.05$.

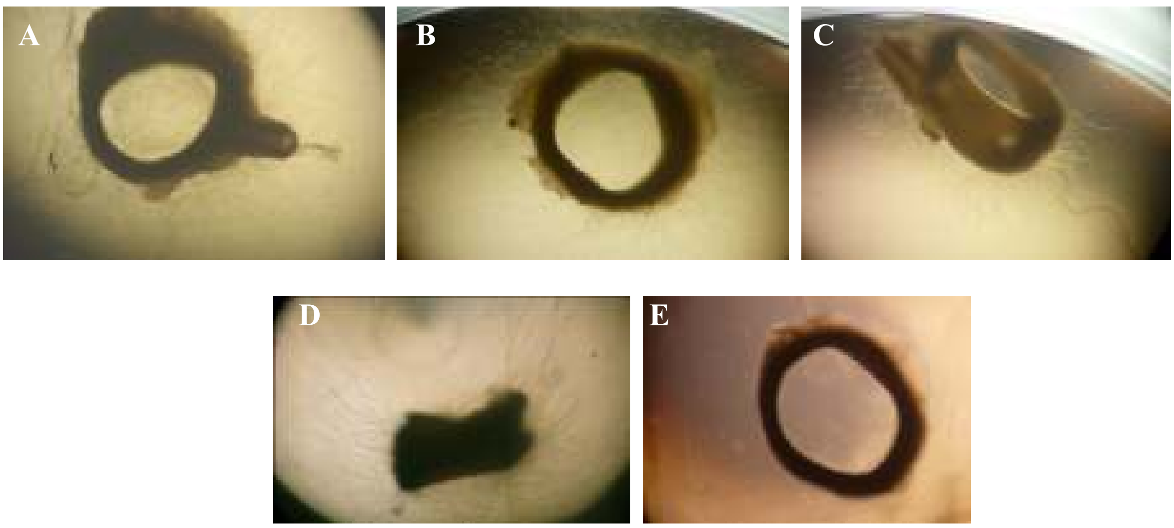


Figure 67. Effect of β-caryophyllene on angiogenesis.
The concentration of caryophyllene used was 1 µM in A, 10 µM in B and 100 µM in C. (D) control (E) suramin. Note extensive capillary growth in A, B &D, reduced capillary growth in C and almost complete inhibition by suramin in E.

4.6. Effect of β-Caryophyllene on Fertility

No statistically significant difference was found for the effect of β-caryophyllene on ovulation, implantation, mid-gestation, late-gestation and normal delivery on any of the studied parameters (Figure 68-71, Table 9-13) including number of implantation sites, adsorption sites, corpora lutea (Figure 72), number and weight of fetuses and newborns and estrogen and progesterone serum levels. In normal delivery experiment, parturition was on day 22-23 of pregnancy in animals of both control and β-caryophyllene- treated group.

Table 9. Effect of β-caryophyllene on ovulation

	Number of implant sites	**Number of adsorption sites**	**Number of corpora lutea**	**Estrogen level (pg/ml)**	**Progesterone level (ng/ml)**
Control, N=7	10.57 ± 1.6	0±0	17.14±4.0	20.1±2.6	111.2±7.8
β-caryophyllene, N=6	9.83 ± 1.1	0±0	18.00±2.9	21.8±2.0	124.8±29.0

Values are mean ±SD. No statistically significant difference was found.

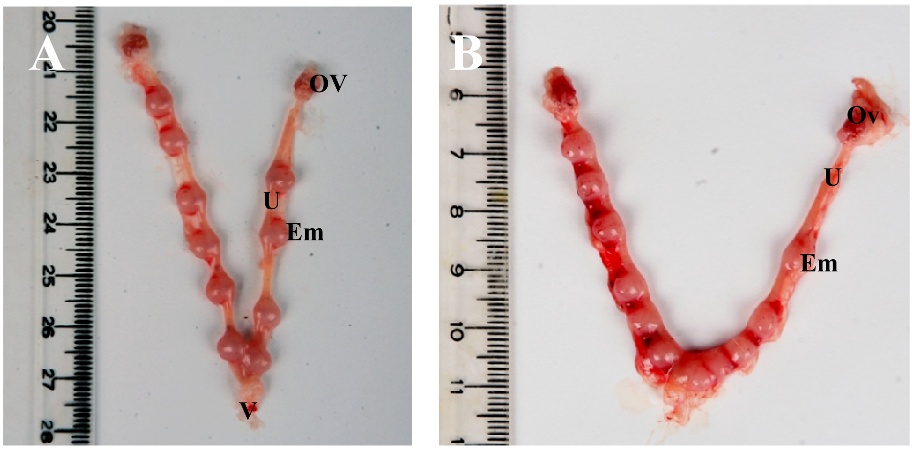


Figure 68. Effect of β-caryophyllene on ovulation.
(A) Uterus of a control rat on day 10 of pregnancy containing 10 embryos and no adsorption sites. (B) Uterus of a rat that received 50 mg/kg caryophyllene at day -3,-2, &-1 before mating. This uterus contains 11 embryos and no adsorption sites. Em: embryo; Ov: ovary; U: uterus; V: vagina.

Table 10. Effect of β-caryophyllene on implantation

Treatment	N	Number of implant sites	Number of adsorption sites	Number of corpora lutea	Estrogen level (pg/ml)	Progesterone level (ng/ml)
Control	7	9.71±1.4	0±0	19.6±2.3 N=5	20.1±2.1	105.4±32.7
β-caryophyllene	6	9.00±1.1	0±0	18.0±5.3	20.3±2.6	97.2±23.8

Values are mean ±SD. No statistically significant difference was found.

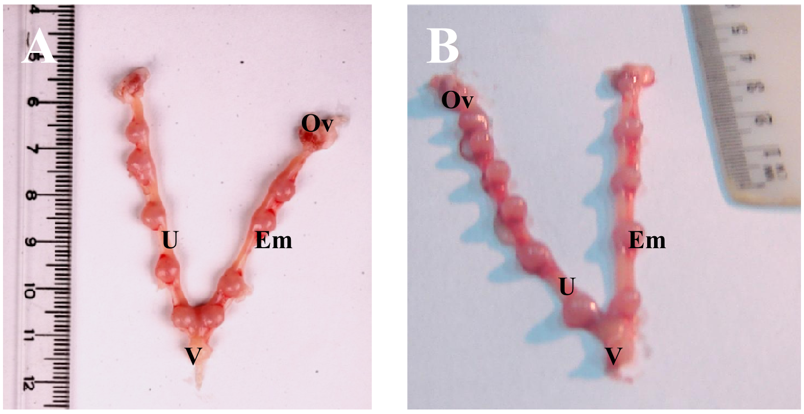


Figure 69. Effect of β-caryophyllene on implantaion

(A) Uterus of a control rat on day 10 of pregnancy containing 9 embryos and no adsorption sites. (B) Uterus of a rat that received 50 mg/kg caryophyllene on 3rd,4th and 5th day of pregnancy. This uterus contains 10 embryos and no adsorption sites. Em: embryo; Ov: ovary; U: uterus; V: vagina.

Table 11. Effect of β-caryophyllene on mid-gestation

Treatment	Number of living fetuses	Number of adsorption sites	Number of corpora lutea	Estrogen level (pg/ml)	Progesterone level (ng/ml)	Weight of fetus (gm)
Control, N=6	9.00±1.2	0.67±0.8	10.33±1.3	39.3±13.8	157.9±13.8	0.235±0.031
β-caryophyllene, N=6	9.50±2.0	1.33±1.1	11.50±1.6	39.2±5.4	130.6±19.9	0.254±0.023

Values are mean ±SD. No statistically significant difference was found.

A **B**

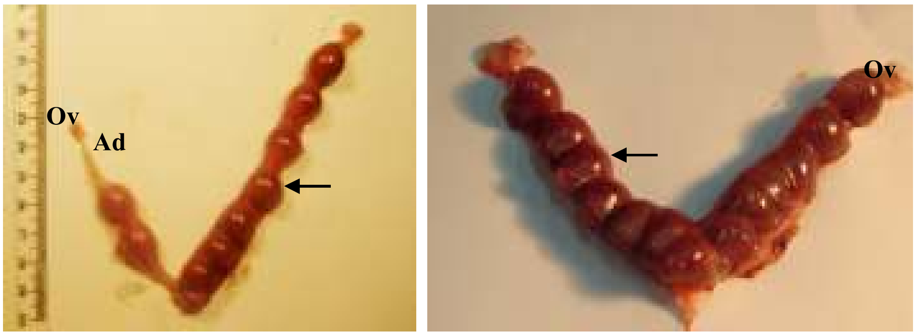


Figure 70. Effect of β-caryophyllene on mid-gestation
(A) Uterus of a control rat on day 16 of pregnancy containing 9 fetuses (arrow) and 4 adsorption sites. (B) Uterus of a rat that received 50 mg/kg caryophyllene on day 13, 14 &15 of pregnancy. This uterus contains 13 fetuses (arrow) and no adsorption sites. Ad: adsorption sites; Em: embryo; Ov: ovary.

Table 12. Effect of β-caryophyllene on late gestation

Treatment	Number of living fetuses	Number of adsorption sites	Number of corpora lutea	Weight of fetus (gm)	Estrogen level (pg/ml)	Progesterone level (ng/ml)
Control, N=6	9.16±1.7	0.67 ±1.2	13.67±3.3	3.30±0.26	31.6±5.9	95.8±11.1
β-caryophyllene, N=6	9.50±1.7	0.33±0.5	11.33±1.2	3.29±0.30	34.0±3.6	77.0±25.4

Values are mean ±SD. No statistically significant difference was found.

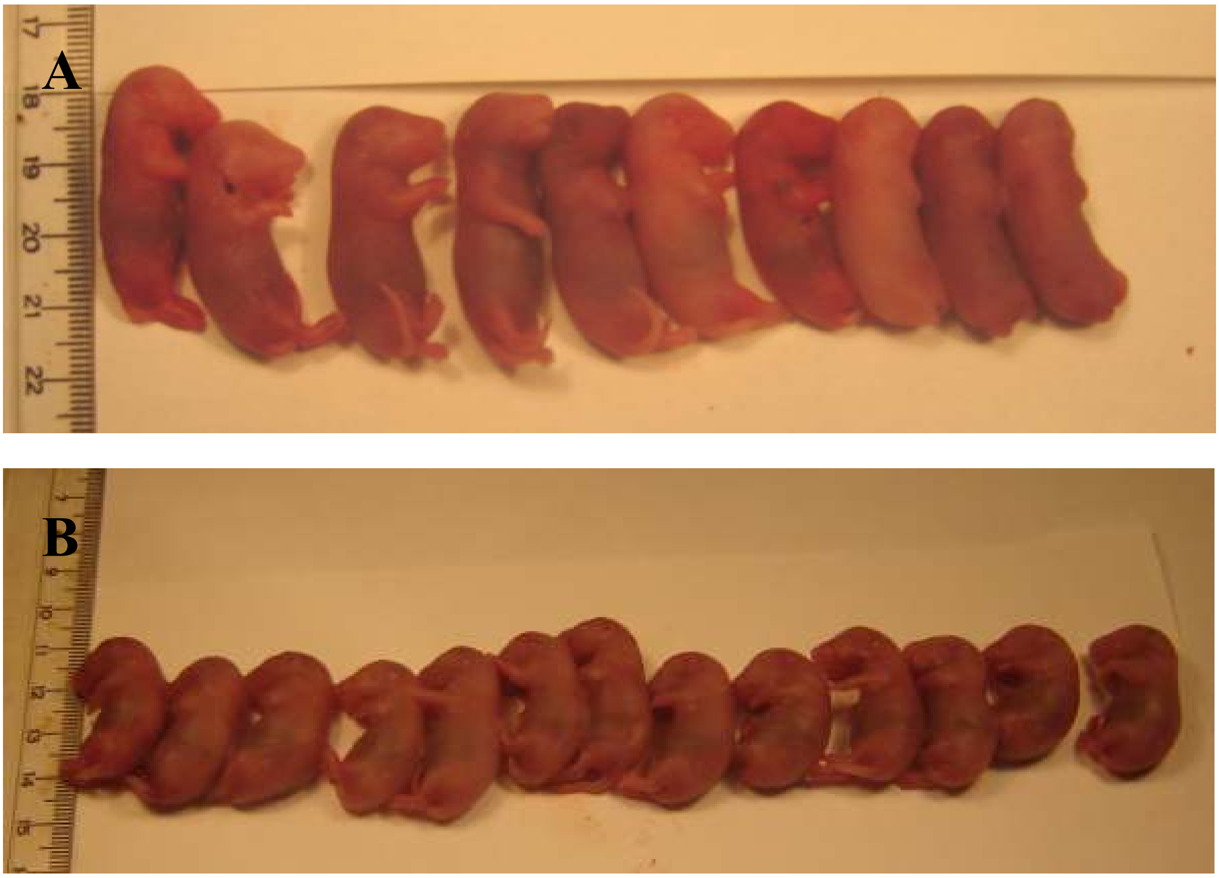


Figure 71. Effect of β-caryophyllene on late-gestation.
(A) Ten living fetuses on day 21 of pregnancy of a control rat. (B) Thirteen living fetuses on day 21 of pregnancy of a rat that received 50 mg/kg caryophyllene on day 18, 19 & 20 of pregnancy. This indicates that caryophyllene does not affect late gestation and had no teratogenic effects on fetuses.

Table 13. Effect of β-caryophyllene on normal delivery

Treatment	Number of living pups	Weight of pup(gm)
Control, N=5	8.20±1.4	4.82±0.65
β-caryophyllene, N=5	7.60±1.3	4.62±0.57

Values are mean ±SD**.** No statistically significant difference was found.

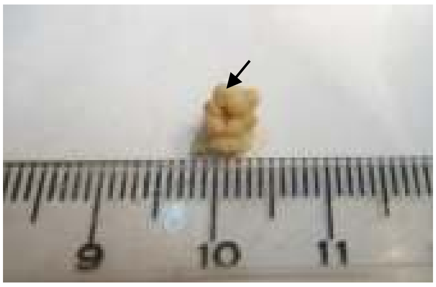


Figure 72. An ovary showing prominent corpora lutea.
This figure shows an ovary with corpora lutea (arrow).

Discussion and Conclusion

5.1. Regression of Endometrial Explants in Oophorectomized Rats

In animals which underwent oophorectomy after cyst development, the cyst disappeared regardless of its original size (Figure 15). Similar results where obtained by Jones (1984) in which removal of the ovaries produced complete regression of growth of the explants in all rats. Furthermore, Cummings and Metcalf (1995) have shown that removal of hormone support by oophorectomy results in very small or no sites at all.

5.2. Is Caffeine a Risk Factor for Endometriosis?

Missmer *et al*. (2004) investigated the relations of lifestyle factors with endometriosis in a prospective cohort study. No association with caffeine intake was found. Similar results were obtained by Pauwels *et al*. (2001). On the other hand, Bérubé *et al*. (1998) reported a positive association between caffeine intake and endometriosis. In fact, there is support for the idea that lifestyle exposures that might raise or lower estrogen levels could affect risk for endometriosis, including an increased risk associated with caffeine use (Cramer and Missmer, 2002; Nagata *et al*., 1998). Furthermore, a significant increase in the risk of infertility due to endometriosis was observed for the upper levels of caffeine intake. The relative risk was 1.9 in women who consumed 5.1–7 g caffeine/month (Grodstein *et al*., 1993). Our results suggest that caffeine does not have a significant effect in treating endometriosis. Also, it does not increase cyst cross sectional area which means that it does not aggravate endometriosis in the rat surgical model used in this study.

5.3.1. Effect of β-Caryophyllene on Regression of Endometrial Explants

In the present study, 10 mg/kg β-caryophyllene caused regression of endometrial implants by 52.5%. This effect may be attributed to the well known anti-inflammatory properties of β-caryophyllene. In rat carrageenan-induced paw oedema model, β-caryophyllene reduced the production of PGE_2, iNOS and COX-2 expression. Its anti-inflammatory activity was comparable to that of dexamethasone, used as positive control drug (Fernandes *et al*., 2007). More recently, it was demonstrated that β-caryophyllene acts also as a potent, selective and nonpsychoactive full agonist for CB_2 receptor *in vivo* thereby exerting anti-inflammatory effect. Interestingly, the lowest oral dose tested (5 mg/Kg) of β-caryophyllene exhibited the most pronounced anti-inflammatory activity (Gertsch *et al.,* 2008).

Many studies have shown that COX-2 inhibition was effective in animal models for the treatment of endometriosis (Dogan *et al.,* 2004, Ozawa *et al*., 2006). To the best of our knowledge, no previous study has investigated the effect of β-caryophyllene on endometriosis. However, it is not clear whether this action is mediated by COX-2 inhibition or CB_2 agonism or any other mechanism. So, more detailed investigations are needed to clarify these aspects.

In a rat carrageenan-induced paw oedema model, β-caryophyllene diminished tumour necrosis factor-α (TNF-α) release (Fernandes *et al*., 2007). Furthermore, it inhibited lipopolysaccharide (LPS)-stimulated TNF-α and IL-1β expression in peripheral blood (Gertsch *et al*., 2008). TNF-α is a key factor in inflammatory processes concerning endometriosis. When neutralized by the recombinant human TNFRSF1a (r-hTBP1, the soluble form of TNF receptor type 1), lesion development diminished in rats (D'Antonio *et*

al., 2000) and in baboons (D'Hooghe *et al*., 2006). Braun *et al* (2002) also implicated TNF-α as the factor in peritoneal fluid from women with endometriosis that enhances proliferation of ectopic endometrial cells (Dmowski and Braun, 2004). This may explain -at least in part- the effect of β-caryophyllene on regression of endometrial explants in our work.

Several investigators have demonstrated a decrease in NK cell cytotoxic activity against autologous and heterologous endometrial cells in women with endometriosis (Vinatier *et al*., 1996). According to Da Silva *et al*., (2007), β-caryophyllene improved natural killer cell activity *in vitro* and *in vivo*.

Several COX-inhibitors and NSAIDs are already prescribed in the clinic as first-line treatment to manage endometriosis caused pain. CB_2 receptor ligands have also been shown to exhibit analgesic effects (Gertsch *et al*., 2008). Since β-caryophyllene possesses COX-2 inhibitory and CB_2 agonistic activity (Fernandes *et al*., 2007; Gertsch, 2008), it is expected that it will have pain alleviating effect in endometriosis. However, according to Bakır *et al*. (2008), β-caryophyllene did not show any analgesic activity at the dosages tested (0.025, 0.05 and 0.1 ml/kg). More investigations are needed to test the analgesic effect of higher doses of β-caryophyllene such as those used in this study.

It was recently shown that CB_2 receptor activation induces apoptosis through *de-novo* ceramide synthesis in colon cancer cells, with tumour necrosis factor (TNF)-α acting as a link between CB receptor activation and ceramide production (Cianchi *et al*., 2008). Our results demonstrated for the first time that β-caryophyllene induced apoptosis in epithelial,

few cells in stroma as well as in endothelial cells of blood vessels *in vivo* (Figure 40). Further studies are needed to explore the apoptotic pathway involved.

5.3.2. Antiangiogenic Effect of β-caryophyllene

Using aortic ring assay, this study has shown for the first time that a high dose of caryophyllene (100 μM) inhibits capillary blood vessel development by 51%. Furthermore, using TUNEL assay, this study illustrated that some endothelial cells exhibited apoptic cell death. This may contribute to the regression in cyst size in the rat surgical model employed here.

Recent studies suggested that angiogenesis plays a pivotal role in the pathogenesis of endometriosis (Taylor *et al.*, 2002). Inflammatory and angiogenic processes seem to lie closely together in many diseases including endometriosis and often the same factors are regulated in both incidents (Eltern, 2008). For example, IL-8 promotes angiogenesis (Strieter *et al.*, 1992) and, together with TNF-α, leads to an increased release of VEGF-A by neutrophils (Na *et al.*, 2006). Another recent study demonstrated that TNF-α leads to a secretion of VEGF-A from infiltrating neutrophils and macrophages in an endometriosis mouse model (Lin *et al.*, 2006). Furthermore, it has been suggested that COX-2 selective inhibitors could be effective in suppressing the establishment and growth of endometriosis - at least partially - through their antiangiogenic activity (Machado *et al.*, 2010).

Different cannabinoid compounds with varying CB1 and CB_2 receptor affinities decreased the formation of new blood vessels. Different mechanisms of action for cannabinoids have been suggested like down-regulation of essential proangiogenic factors such as VEGF,

placental growth factor and angiotensin II accompanied by dephosphorylation of VEGF receptors 1 and 2 (Oesch and Gertsch. 2009). Since β-caryophyllene exhibits potent CB_2 receptor agonistic activity (Gertsch *et al.*, 2008), then it may exert its anti-angiogenic activity through this mechanism.

5.3.3. Effect of β-Caryophyllene on Fertility

It was clear from the results of fertility experiments that β-caryophyllene (50 mg/kg, i.p) was completely safe during early, middle and late pregnancy. Furthermore, it has no statistically significant effect on ovulation or normal delivery. In fact, β-caryophyllene is an FDA-approved food additive (Gertsch *et al.*, 2008) and it is apparently a non-toxic compound with no genotoxic or cytotoxic effect *in vivo* (Molina-Jasso *et al.*, 2009).

According to Gaytan *et al.* (2006), treatment with NSAIDs, either non-selective or selective COX-2 inhibitors, consistently impaired ovulation. This illustrates the essential role of COX-2/prostaglandins in the ovulatory process. A study conducted by Espey (1983) has demonstrated that ovulation was completely inhibited by diclofenac and indomethacin. However, it was partially inhibited by fenoprofen, and unaffected by aspirin, acetaminophen, or dexamethasone. This suggests that any potent NSAID can inhibit ovulation provided it adequately reduces PG production. The anti-inflammatory agent must completely abolish the preovulatory elevation in PGs in mature follicles in order to totally inhibit ovultion. Similarly, Efstathiou *et al.* (2005) has demonstrated that the selective COX-2 inhibitor celecoxib did not have a negative effect on the estrus cycle in mice, while inhibiting growth of endometriotic lesions.

In conclusion, β-caryophyllene caused a significant reduction in cyst cross sectional area and produced apoptosis in endometrial explants without interfering with pregnancy or ovulation. Further studies are needed to explore its exact mechanism of action.

5.4. Effect of Citronellol on Regression of Endometrial Explants

Despite its anti-inflammatory activity *in vitro* (Abe *et al*., 2003), citronellol produced no statistically significant regression in endometriotic implants *in vivo*.

5.5. Effect of NDGA (Masoprocol) on Regression of Endometrial Explants

Oxidative stress has been proposed as a potential factor in the pathophysiology of endometriosis (Gupta *et al*., 2006). Therefore, it has been suggested that effective antioxidant therapy for patients with endometriosis would be helpful. Interestingly, significant clinical improvements have been demonstrated when antioxidants were given as adjuvant therapy (Balasch *et al*., 1997; Steinleitner *et al*., 1991). For example, vitamin C and E therapy reduced pelvic pain in women with endometriosis (Kavtaradze *et al*., 2003). More recently, it has been demonstrated that treatment with melatonin, a potent antioxidant and free radical scavenger, effectively regresses endometriotic explants in a rat model (Guney *et al*., 2008). The anti-oxidant properties of NDGA are well known (Floriano-Sánchez *et al*., 2006). In addition NDGA is a lipoxygenase inhibitor (Fujiwara *et al*., 1998). This may explain, at least in part, its effectiveness in regressing endometriotic implants reported for the first time in this study.

Results of the present study illustrate that NDGA produces apoptosis in both epithelial and stromal components of endometriotic cysts (Figure 41), though the effect on epithelial cells

was more pronounced. Induction of apoptosis by NDGA in tumor cells was reported (Hausott *et al*., 2003). However, the exact apoptotic pathway involved in our study is not clear.

Several studies have demonstrated the role of ERβ in the pathophysiology of endometriosis. It has been suggested that the upregulation of ERβ in this disease may have an anti-inflammatory role (Bukulmez *et al*., 2008). A selective ERβ agonist, ER-041 caused complete lesion regression in 40-75% of the animals (Harris *et al*., 2005). The effect of NDGA on endometriosis demonstrated here may be attributed, at least partially, to its ERβ agonistic activity.

5.6.1. Effect of Primaquine on Regression of Endometrial Explants

Primaquine is reported here for the first time to be effective in decreasing cyst cross sectional area in rats. This effect may be due to the well known anti-inflammatory activity of primaquine (Goldring and Nemaorani, 1999; Scherbel, 1983; Steck *et al*., 1952).

In this study, it has been shown that primaquine induces apoptosis in some cells in endometriotic implants. This may contribute to its anti-endometriotic effect. According to Noel *et al*. (2007), primaquine causes transcriptional alterations in the apoptosis, cell cycle as well as oxidative stress pathways in liver cells *in vivo*. Whether similar effects on gene transcription occur in a rat endometriosis model is not clear. So, more investigations are needed to explore this aspect and the specific apoptotic pathway involved.

5.6.2. Effect of Primaquine on Fertility

Primaquine is contraindicated during pregnancy because of the possibility of inducing haemolysis and methaemoglobinaemia in the fetus. It was recommended that pregnant women who require radical treatment for vivax malaria should receive primaquine after delivery (Taylor and White, 2004).

5.7.1. Effect of Sunitinib on Regression of Endometrial Explants

In this study, the tyrosine kinase inhibitor sunitinib (10 mg/Kg) caused complete cyst regression in 50% of the animals with an average reduction in the cross sectional area of the cyst of 80.3 %. To the best of our knowledge, this is the first time sunitinib is reported to cause such an effect. Similar anti-endometriotic effects were obtained using the tyrosine kinase inhibitor imatinib. Imatinib decreased proliferation and transmesothelial migration by endometrial cells in an *in vitro* model (Griffith *et al*., 2010). Also, it resulted in 35% reduction in number of endometriotic lesions formed in a murine model of endometriosis (Jensen *et al*., 2008).

Ngo *et al*. (2010) provided evidence that protein kinase inhibitors can control endometriotic cell proliferation *in vitro* and endometriosis progression *in vivo*. Treating endometriotic cells with A771726, PD98059 or U0126 significantly decreased cellular proliferation *in vitro*. *In vivo*, A771726, leflunomide, PD98059, and U0126 controlled the growth of endometriotic implants in the mouse model of endometriosis. So, it was suggested that protein kinase inhibitors could be new candidates to treat endometriosis.

In this study, it was observed that fat adhesions were almost absent in animals treated with 10 mg/Kg sunitinib (Figure 16). This agrees with the findings of Kim *et al*. (2008) who demonstrated that sunitinib significantly reduces adhesion formation in a murine model.

Sunitinib malate is a yellow to orange powder. The renal system is the primary route of its excretion (Patyna *et al*., 2008). Yellow coloration of hair and urine was observed in animals treated with 10 mg/Kg sunitinib for 21 days (Figure 17). Patyna *et al*. (2008) found similar yellowish discoloration of the fur in most rats given the doses (5 and 15 mg/kg/day) in thirteen-week study. Also, signs of discolored (dark yellow) urine occurred in six-month study in rats given $\geq$ 0.3 mg/kg/day. It was concluded that this yellow discoloration of skin, fur, and urine was caused by the yellow color of the test drug or its metabolite(s).

Upon treatment, sunitinib interfered with healing of the surgical wound (Figure 17). This was expected since neoangiogenesis is essential for wound healing (Patyna *et al*., 2008) and sunitinib had well known anti-angiogenic effects (Roskoski, 2007).

It was demonstrated -for the first time- in the present study that sunitinib induces apoptosis in endometriotic explants in a surgical rat model. Apoptosis was observed in the lining endometrium, some cells in the stroma and in endothelial cells of blood vessels (Figure 44). Recently, it was reported that inhibition of signal transducer and activator of transcription 3 (STAT3) by sunitinib induced renal cell carcinoma tumor cell apoptosis and growth arrest prior to tumor vasculature collapse (Xin *et al*., 2009). Furthermore, it was shown that sunitinib induces tumor cell apoptosis and growth arrest in a primary culture (VC312) and an established cell line (Daoy) of medulloblastomas through the inhibition of STAT3 and AKT signaling pathways (Yang *et al*., 2010). It is not clear if similar mechanism is

involved in apoptosis produced by sunitinib in this study. So, more investigations are needed.

5.7.2. Antiangiogenic Effect of Sunitinib

It was suggested that inhibition of angiogenesis may lead to regression of endometriotic implants (Onalan, *et al*., 2009). Sunitinib inhibition of VEGFRs and PDGFRs expressed by vascular or perivascular endothelial cells is predicted to produce anti-angiogenic effects. A recent study by Yao *et al*. (2006) demonstrated that the dual inhibition of VEGFR and PDGFR with sunitinib is associated with greater antiangiogenic effects compared with selective inhibition of VEGFR or PDGFR alone (Christensen, 2007).

In our experiment, sunitinib caused cyst regression after twenty one days of treatment. It is expected that it caused this regression due, at least in part, to its anti-angiogenic activity. Apoptosis of endothelial cells was also evident using TUNEL assay (Figure 44). According to Baffert *et al*. (2006), the time course of capillary regression begins within two weeks of treatment, with the loss of vessel patency, intraluminal fibrin deposition, and cessation of blood flow. Apoptosis and the loss of endothelial cells follow. Pericytes either retract from regressing endothelial cells onto adjacent normal vessels or undergo regression themselves, while empty sleeves of basement membrane remain temporarily at sites of endothelial cell regression and assist in capillary regrowth (Mancuso *et al*., 2006). Electron micrographs of small blood vessels in stroma of endometriotic tissues in sunitinib-treated group have not revealed a clear abnormality in their structure (Figure 66).

5.7.3. Effect of Sunitinib on Fertility

Sunitinib-treated rats and monkeys had decreased ovarian and uterine weights. Also, impaired formation of corpora lutea and uterine development during the estrus cycle were observed in rats. Similarly, decreased ovarian follicular development and uterine endometrial atrophy were also evident in monkeys. However, ovarian effects at the end of the recovery period were completely reversed in the thirteen-week and nine-month monkey studies while persisted only with the highest dose (15 mg/kg/day) in rats (Patyna *et al.*, 2008). In our study, the dose of sunitinib used was 10 mg/kg/day. So, it is expected to have reversible reproductive effects.

At doses of ≥5 mg/kg/day in rats and 5 mg/kg/day in rabbits, sunitinib produced a decrease in the number of live fetuses and increase in the number of resorptions and post-implantation/complete litter losses. The no-observed-adverse-effect level (NOAEL) for sunitinib was 1–5 mg/kg/day for maternal toxicity, and 3 mg/kg/day for developmental toxicity in rats; 1 and 0.5 mg/kg/day, respectively, in rabbits (Patyna *et al.*, 2009).

5.8.1. Effect of Theophylline on Regression of Endometrial Explants

The percentage regression of endometrial explants in theophylline-treated group was 52.0% and 65.5% for 10 mg/kg/day and 15 mg/kg/day doses, respectively. To the best of our knowledge, this study represents the first report for such an effect. However, the mechanism of action is not clear yet. This activity could be attributed to the anti-inflammatory effect of theophylline (Barnes, 2010) or to another mechanism such as anti-angiogenic activity. Theophylline and corticosteroids were reported to have a synergistic

effect in repressing inflammatory gene expression (Barnes, 2010). So, their combination may have a pronounced effect on treatment of endometriosis.

In human leukocyte-induced angiogenesis test, theophylline significantly decreased the angiogenic activity (Skopinski *et al.*, 1993). The structurally related methylxanthine theobromine exhibited antiangiogenic properties through A_2 receptors antagonism (Barcz *et al.*, 2000). Since theophylline is a potent inhibitor of A_2 receptors (Pauwels and Joos, 1995), it is expected to exhibit such activity. However, it is not clear from the results of this study if theophylline exerts its anti-endometriotic action in rat model of endometriosis through its anti-angiogenic activity. Electron micrographs of blood vessels in cysts of theophylline-treated group have not revealed a clear abnormality in their structure (Figure 65).

Theophylline promoted apoptosis in neutrophils (Yasui *et al.*, 1997), eosinophils (Adachi *et al.*, 1996; Ohta and Yamashita, 1999) and T lymphocytes (Ohta and Yamashita, 1999). A reduction in the antiapoptotic protein Bcl-2 was suggested (Chung *et al.*, 2000). This study demonstrates for the first time that theophylline produces apoptosis in endometriotic implants. More investigations are needed to discover the apoptotic pathway involved.

5.8.2. Effect of Theophylline on Fertility

After 19 weeks of oral administration of theophylline, a crossover mating indicated that females and males were adversely affected by theophylline, as judged by the decreased percentage of pups born alive, the decreased live pup weight, and the decreased number of live pups per litter relative to matings within the control group (Morrissey *et al.*, 1988).

Another study conducted by Lindström (1990) reported the NOAELs for maternal toxicity to be 218 mg/kg for rats and 282 mg/kg for mice. On the other hand, NOAELs for developmental toxicity were 124 mg/kg for rats and 282 mg/kg for mice. In fact these NOAELs are approximately 10- to 30-fold greater than doses required to maintain humans on serum theophylline concentrations that are clinically useful. This may explain why theophylline is considered effective and safe for use during pregnancy in humans (Katz and Sheiner, 2008). The doses used in the present study were 10 mg/kg and 15 mg/kg which are much less than the doses caused reproductive toxic effects mentioned above.

In conclusion, the mechanism of action by which theophylline exerts its effect in endometriosis should be studied in details. The anti-endometriotic activity of other structurally related compounds like theobromine deserves evaluation. The future may bring a resurgence of theophylline working synergestically with other drugs; thereby decreasing the dose and subsequently the side-effects of theophylline. Coming research may also introduce a new structural modification as an improvement on the parent compound.

5.9. Effect of Thymoquinone on Regression of Endometrial Explants

Despite the well known biological activities reported in various studies as in El-Mahmoudy *et al*. (2002) and Houghton *et al.* (1995), thymoquinone produced no regression in endometriotic implants in this study. A possible explanation -though not confirmed at our laboratory- is that thymoquinone was biotransformed to dihydrothymoquinone. According to Al-Johar *et al*. (2008), no thymoquinone was detected in serum samples upon oral administration. So, it was suggested that it has been biotransformed to

dihydrothymoquinone by DT-diaphorase in the liver (Nagi *et al.*, 1999; Mansour *et al.*, 2002).

5.10. Effect of Vitamin D on Regression of Endometrial Explants

The immune system has been advocated to play a critical role in the pathogenesis of endometriosis. It has been shown that lymphoid function is altered and natural killer cell cytoxicity is hampered in this disease (Lebovic *et al*., 2001; Vigano *et al*., 2006a). The impaired immune-mediated clearance of endometrial fragments refluxed into the pelvic peritoneum during menstruation has been hypothesized to permit these cells to survive and implant in ectopic sites (Osuga *et al*., 2011). The hypothesis tested in the present study was that vitamin D may influence the development of endometriosis. Using a rat surgical model for endometriosis it was demonstrated that vitamin D reduced cyst cross sectional area by 51.1%. To the best of our knowledge, this is the first report demonstrating that vitamin D has such an effect in an animal model of the disease. However, the exact mechanism by which it functions needs more investigation.

In the present study, vitamin D produced apoptosis in endometriotic implants. Several authors have reported that vitamin D can induce apoptotic death in different tumor cells (Diaz *et al*., 2000; Welsh, 1994).

The recent elucidation of the crystal structure of the vitamin D receptor bound to its natural ligand will facilitate the development of 1,25-$(OH)_2D_3$ analogs with greater potency, lower calcemic liability and increased tissue specificity. Also, selective immunoregulatory properties may be enhanced (Mathieu and Adorini, 2002).

5.11. Interpretation of Light and Electron Microscopic Results

In the oophorectomized rats, only small, degenerating glands were encountered (Figure 21). This agrees with the results of Katsuki *et al.* (1998) who reported that the epithelium was atrophic in oophorectomized rats.

Spiral arterioles were seen in tissue sections of oophorectomized rats (Figure 21 A). It is well-established that spiral arterioles reach their maximum tortuosity towards the end of the secretory phase (Ferenczy, 1987), at the time when the endometrium is undergoing regression. It has been suggested that the process of endometrial regression plays a role in increasing the coiling of the spiral arterioles by forcing them to shorten as the endometrium becomes thinner (Rogers, 1996).

In the present study, fibrosis was encountered in histologic sections of endometriotic explants of the active compounds along with a pronounced decrease in the number of glands (Figure 25, 28, 31, 34, 36). Ultrastructural study revealed the presence of collagen fibers in the stroma of all studied compounds (Figure 48-50). However their density varied. Numerous studies have reported the presence of fibrosis in regressing endometriotic cysts (Bergqvist *et al*., 1985; Styer *et al*., 2008). For example, disruption of leptin signaling resulted in a predominance of fibrosis, with a paucity of endometrial glandular epithelium and marked stromal obliteration compared with control (Styer *et al*., 2008).

It has been demonstrated that aromatase, the enzyme that catalyzes the conversion of C_{19} steroids to estrogens, is expressed in endometriotic stromal cells (Bulun *et al*., 1999). So, endometriotic glands are sustained by their own endometrial stroma (Rossi *et al*., 2000). According to Bergqvist *et al*. (1985) the growth of fibrotic tissue toward the central part of

the graft leads to the degradation of glands deprived of surrounding stroma. This may explain the decrease in the number of glands associated with fibrosis obtained in this study.

Several lines of evidence suggest that mast cells may participate in fibrotic processes. For example, some chronic allergic conditions in which repeated stimulation of mast cells has been demonstrated are associated with the development of fibrosis (Metcalfe, 1997). In endometrial cysts, most mast cells were concentrated around blood vessels and the interstitium with fibrosis while few mast cells were localized in the stroma near regions of endometrial gland-like structures (Fujiwara *et al*., 2004). In the present study, few mast cells were encountered in the stroma and many were concentrated in close proximity to small blood vessels (Figure 37) at the border between the skeletal muscles and the stroma. However, these were not the main sites of fibrosis.

Light microscopic studies revealed degranulation of mast cells in the control group as well as under the action of the different active compounds (Figure 37-38). Ultrastructural studies (Figure 52-56) demonstrated that most mast cell granules have reduced electron density. Singh *et al*. (1999) suggested that this loss of electron density of granules indicates secretory activity.

Mast cells contain granules that store preformed effector molecules such as histamine, serotonin, serine proteases, exopeptidases and neuropeptides (Stevens and Austen 1989). When activated, mast cells release these granules in a process referred to as compound exocytosis or degranulation (Dvorak, 1991). It has been suggested that the released mediators alone or in combination act on the vasculature, smooth muscle, connective tissue, mucous glands, and inflammatory cells. These mediators are responsible for the clinical signs and symptoms of allergic responses (D’Cruz, and Uckun, 2007).

An association between tissue eosinophilia and fibroplasias has been demonstrated. Eosinophil extracts stimulated fibroblast proliferation, suggesting that an eosinophil product directly causes fibrosis (Noguchi *et al*., 1992). Furthermore, human eosinophils stimulated DNA synthesis and matrix production in dermal fibroblasts (Birkland *et al*., 1994). According to Blumenthal, *et al*. (2000), degranulating eosinophils were present in large numbers in the fibrous regions of endometriosis. So, they suggested that eosinophils are involved in general tissue remodeling and wound healing rather than in a tissue-directed immune response. Using Western blot and immunohistochemistry, the same authors provided evidence that eosinophils are implicated in fibrotic process in endometriosis. Furthermore, they reported the presence of free red granules suggestive of disintegrated eosinophils. Our findings suggest that eosinophils lie in close proximity to fibrotic tissue, which was most commonly encountered directly under the luminal epithelium.

In the present study, hemosiderin-laden macrophages were commonly encountered in endometriotic explants (Figure 23-25, 31, 35). According to Blumenthal *et al*. (2000) healed foci of endometriosis usually consist only of fibrous tissue and hemosiderin-laden macrophages. Furthermore, the scoring system used by Ngo *et al*. (2010) regarded the presence of hemosiderin a good prognostic factor as apposed to the presence of blood. In this scoring system, pathology Zero score was assigned to receded lesion with stromal fibrosis, hemosiderin, and absence of glandular structure and score three to active lesion with fresh blood, profuse stromal cellular infiltration, and developed glandular organization.

Another histologic sign of regression in endometriotic cysts is the atrophy of the epithelial tissue. For example, in histologic sections from rats treated with high dose of atorvastatin and leuprolide acetate marked atrophy of epithelial tissue has been reported (Oktem *et al.* 2007). Similarly metformin caused regression of epithelial tissue (Oner *et al.*, 2010). However, in all studied compounds, except some specimens of theophylline-treated group (Figure 33), no atrophy in luminal epithelial component was observed.

Ultrastructural studies of eosinophils of the control group revealed the presence of granules with dark crystals and granules with light crystals (Figure 57). According to Miller *et al.* (1966), specific granules in an eosinophil leukocyte show dense cores of various shapes embedded in a less dense matrix. These granules are bound by a single unit-membrane. The core of eosinophil granules is a crystal which has a cubic lattice. Major basic protein, the predominant cationic protein of human eosinophil specific granules, is stored within crystalloid cores of these granules. Secretion of this protein contributes to the immunopathogenesis of varied diseases (Melo *et al.*, 2009).

Electron micrographs of eosinophils from endometriotic tissue of theophylline-treated group showed only granules with dark crystals (Figure 61). To the best of our knowledge, this is the first report about such an effect. Theophylline inhibited IL-5–dependent eosinophil survival by means of apoptosis (Ohta and Yamashita, 1999). Hatzelmann *et al.* (1995) reported that theophylline inhibited eosinophil degranulation. This may explain the presence of only granules with dark crystals in eosinophils in theophylline-treated group.

Infiltration of stroma by chronic inflammatory cells, including lymphocytes and macrophages was reported by several authors (e.g. Haber *et al.*, 2009). According to

Jolicoeur *et al*. (2001), endometrial cells express the chemotactic factor of the monocytes (MCP-1), responsible for the grouping and activation of macrophages. In the present work, the presence of macrophages (Figure 63) and plasma cells in the stroma has been demonstrated using electron microscopy.

5.12. Recommendations

This study illustrated for the first time that β-caryophyllene, nordihydroguaiaretic acid (masoprocol)**,** primaquine, sunitinib (sutent), theophyllene, and vitamin D are effective in treating endometriosis in a rat surgical model. However, the following aspects need detailed investigations:

1. Studying the effect of structurally-related compounds e.g theobromine on endometriosis.
2. Ultrastructural studies of the effect of the active compounds on endometriotic explants. Also, the mechanism by which they exert apoptosis needs more clarification.
3. Studying the impact of the effective compounds on immunological parameters such as NK cell activity and TNF secretion in endometriosis.
4. Investigating the role of eosinophils, mast cells and pericytes in causing fibrosis in endometriosis.
5. Studying the effect of the active compounds in primates and tissue culture of human endometriotic tissue.
6. Finally, since most of the studied compounds are already approved drugs, clinical studies can be conducted to evaluate their effect in treating endometriosis in human subjects.

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Appendices

Appendix I

Hematoxylin and Eosin Staining Procedure

Mayer's Hematoxylin Solution

Constituents: Hematoxylin (1.0 g/l), sodium iodate (0.2 g/l), aluminum ammonium sulfate.12 H_2O (50 g/l), chloral hydrate (50 g/l), citric acid (1 g/l) and 1L distilled water.

Method: Add the haematoxylin, potassium alum and sodium iodate to the distilled water and dissolve by warming and stirring, Add the chloral hydrate and citric acid and boil the mixture for 5 minutes. Cool and filter if necessary. The solution is then ready for use.

Eosin-Phloxine B Solution

Eosin Stock Solution:
Eosin Y, 1 g
Distilled water, 100 ml
Mix to dissolve.

Phloxine Stock Solution:
Phloxine B, 1 g
Distilled water, 100 ml
Mix to dissolve.

Eosin-Phloxine B Working Solution:
Eosin stock solution, 100 ml
Phloxine stock solution, 10 ml
Ethanol (95%), 780 ml
Glacial acetic acid, 4 ml
Mix well.

1% Lithium Carbonate Solution (Saturated):
Lithium carbonate 1.0 g
Distilled water 100 ml
Mix well.

Staining Procedure

1. Deparaffinize sections, 2 changes of xylene, 10 minutes each.
2. Re-hydrate in absolute alcohol, 95% alcohol and then 70% alcohol (20 dips each).
3. Wash briefly in distilled water.
4. Stain in haematoxylin solution for 15 minutes.
5. Wash in tap water.
6. Differentiate in 1% lithium carbonate (10 dips).
7. Wash in tap water.
8. Counterstain in eosin solution for 5 minute.
9. Dehydrate through 70% alcohol (10dips), 95% alcohol (20 dips) and absolute alcohol (20dips).
10. Clear in 2 changes of xylene (5-10 dips each).
11. Mount with D.P.X.

Results

Nuclei should be blue, cytoplasm pink to red.

Appendix II

Masson's Trichrome Staining Protocol

Fixation: 10% formalin.

Section: Paraffin sections at 5 um.

Preparation of solutions:

Weigert's Iron Hematoxylin Solution:

Stock Solution A:
Hematoxylin ----------------------------- 1 g
95% Alcohol ---------------------------- 100 ml

Stock Solution B:
29% Ferric chloride in water --------- 4 ml
Distilled water ------------------------ 95 ml
Hydrochloric acid, concentrated ---- 1ml

Weigert's Iron Hematoxylin Working Solution:
Mix equal parts of stock solution A and B. This working solution is stable for 3 months

Biebrich Scarlet-Acid Fuchsin Solution:
Biebrich scarlet, 1% aqueous --------- 90 ml
Acid fuchsin, 1% aqueous --------------10 ml
Acetic acid, glacial --------------------- 1 ml

Phosphomolybdic-Phosphotungstic Acid Solution:
5% Phosphomolybdic acid ------------- 25 ml
5% Phosphotungstic acid -------------- 25 ml

Aniline Blue Solution:
Aniline blue ------------------------------- 2.5 g
Acetic acide, glacial --------------------- 2 ml
Distilled water --------------------------- 100 ml

1% Acetic Acid Solution:
Acetic acid, glacial ----------------------- 1 ml
Distilled water ---------------------------- 99 ml

Procedure:

1. For formalin fixed tissues deparaffinize in xylene and rehydrated through 100% alcohol, 95% alcohol 70% alcohol.
2. Wash in distilled water.
3. Stain in Weigert's iron hematoxylin working solution for 10 minutes.
4. Rinse in running warm tap water for 10 minutes.
5. Wash in distilled water.
6. Stain in Biebrich scarlet-acid fuchsin solution for 10-15 minutes. Solution can be saved for future use.
7. Wash in distilled water.
8. Differentiate in phosphomolybdic-phosphotungstic acid solution for 10-15 minutes or until collagen is not red.
9. Transfer sections directly (without rinse) to aniline blue solution and stain for 5-10 minutes. Rinse briefly in distilled water and differentiate in 1% acetic acid solution for 2-5 minutes.
10. Wash in distilled water.
11. Dehydrate very quickly through 95% ethyl alcohol, absolute ethyl alcohol (these step will wipe off Biebrich scarlet-acid fuchsin staining) and clear in xylene.
12. Mount with D.P.X.

Results:

Collagen --------------------------------------- blue
Nuclei -- black
Muscle, cytoplasm, keratin ------------------- red

Appendix III

Toluidine Blue Staining Protocol for Mast Cells

Fixation: 10% Formalin.

Sections: paraffin sections at 5 um.

Solutions and Reagents:

Toluidine Blue Stock Solution:

Toluidine blue --------------------------- 1 g
70% alcohol ------------------------------ 100 ml
Mix to dissolve.

Sodium Chloride (1%):

Sodium chloride --------------------------- 1 g
Distilled water ----------------------------- 100 ml
Mix to dissolve (make this solution fresh each time). Adjust pH to 2.0~2.5 using glacial acetic acid or HCl.

Toluidine Blue Working Solution (pH 2.0~2.5):

Toluidine blue stock solution -------------- 10 ml
1% Sodium chloride, pH 2.3 --------------- 90 ml
Mix well. The pH should be around 2.3 and less than 2.5
Make this solution fresh and discard after use.

Procedure:

1. Deparaffinize and hydrate sections to distilled water.
2. Stain sections in toluidine blue working solution for 10 minutes.
3. Wash in distilled water, 3 changes.
4. Dehydrate quickly through 95% and 2 changes of 100% alcohol (10 dips each since stain fades quickly in alcohol).
5. Clear in xylene or xylene substitute, 2 changes, 3 minutes each.
6. Mount with D.P.X.

Results:

Mast cells -------------------------- violet/red purple.
Background ------------------------- blue.

Appendix IV
Preparation of Specimens for Electron Microscopy

Preparation of Karnovsky Fixative

1. Sodium Cacodylate Buffers 0.4 M

Sodium cacodylate	85.6 gm
D.W	1000 ml

2. Sodium Cacodylate Buffers 0.1 M

0.4 M sodium cacodylate	25ml
1% Calcium chloride	10 drops
0.2M hydrochloric acid	4 ml
D.W	up to 100ml
pH 7.4	

3. 8% Paraformaldehyde solution

Paraformaldehyde	8 gm
D.W	100 mL
10N NaOH	12 drops

Heat to 60°C on a stirrer plate. When moisture forms on the sides of flask, add sodium hydroxide and stir until the solution clears. Cool solution and then filter.

4. Karnovsky Fixative

8% Paraformaldehyde	25 mL
25% glutaraldehyde	10 ml
Sucrose	3 gm
0.1M Na cacodylate buffer	up to 50 ml

Infiltration and Embedding Medium (Spurr's medium)
This medium was prepared using the following:
10 gm venyl cyclohexane dioxide (ERL-2406)
4 gm diglycidyl ether polypropylene glycol (D.E.R 736).
26 gm neonyl succinic anhydride.
0.4 ml of dimethyl aminoethanol (D.M.A.E).
The solution was mixed gently using a magnetic stirrer for 30 minutes.

Preparation of blocks
Pieces of tissue were cut into about $1mm^3$ and immersed in Karnovsky's fixative for 2 hours. The samples were then washed with sodium cacodylate buffer for 30 minutes (3 changes), and then post fixed with 1% OsO_4 in D.W for one hour. Then, samples were washed again 3 times (10 minutes each) with sodium cacodylate buffer. Dehydration was done using a graded series of acetone (30%, 50%, 70%, 95% and 100%). Samples were immersed in each concentration for 5 minutes and twice in 100% (10 minutes). Then, the samples were infiltrated using 50% Spurr medium in acetone for 2 hours, followed by 2

changes of pure Spurr medium and left overnight with continuous rotation. The samples were them embedded in pure Spurr's medium and left in an oven at 60ºC for 48 hours.

Sectioning and Staining

Silver-gold sections (70-90 nm thick) were cut with ultracut E Reichert Tung ultramicrotome and stained with aqueous uranyl acetate (30 miutes) / Renyold's lead citrate (15 miutes) and viewed using Zeiss electron microscope 10 CR/ Yarmouk University, Jordan.

Appendix V

Composition of Buffers and Solutions Used in TUNEL Assay

20X SSC
87.7g NaCl
44.1g sodium citrate
Dissolve in 400ml of deionized water. Adjust pH to 7.2 with 10N NaOH and bring volume to 500ml.

2X SSC
Dilute 20X SSC 1:10 with deionized water before use.

4% paraformaldehyde solution
Weigh out 4g paraformaldehyde in a fume hood, add PBS and bring to 100ml. Dissolve by heating the closed bottle in a water bath at 65°C for 2 hours. Store the solution at 4°C, where it is stable for at least 2 weeks.

Equilibration Buffer
200mM potassium cacodylate (pH 6.6 at 25°C)
25mM Tris-HCl (pH 6.6 at 25°C)
0.2mM DTT
0.25mg/ml BSA
2.5mM cobalt chloride

Proteinase K buffer
100mM Tris-HCl (pH 8.0)
50mM EDTA

تقييم فعالية بعض المُعدلات المناعية وبعض مضادات السرطان كعلاجات مُحتملة للإنتباذ البطاني الرحمي

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مُلخـــــص

يعتبر مرض الإنتباذ البطاني الرحمي أحد أكثر الأمراض النسائية شيوعاً. العلاجات المتوفرة حالياً غير مُرضية بسبب تأثيراتها الجانبية وكذلك تأثيرها السلبي على الخصوبة. تهدف هذه الدراسة إلى اكتشاف أدوية جديدة لهذا المرض بناءً على الفهم الحديث لآلية حدوثه والتقدم الذي تم في فهم دور جهاز المناعة وفي التشابه بين هذا المرض والسرطان. تمت دراسة تأثير تسع مركبات على تراجع المرض بعد إحداثه جراحياً في الجرذان. قلل الكاريوفلين (10ملغم/كغم و30ملغم/كغم), وحـامض نوردايهيدروجويارتيك (مازوبروكول) (10ملغم/كغم), وبريماكوين (10ملغـم/كغـم), وسـنيتينيب (سيوتنت) (10ملغم/كغم), وثيوفلين (10ملغم/كغم و15ملغم/كغم), وفيتامين د (42مايكروغم/كغـم) مساحه المقطع العرضي للكيس بنسـبة 52,5%, 41,0%, 45,4%, 43,1%, 80,3%, 52,0%, 65,5% و 51,1% على الترتيب. من جهة أخرى, لم تعـط مركبـات الكـافيين, وسـيترونيللول وثيموكوينون أي تأثير ملحوظ. نسيجياً, لوحظ حدوث تليف واسع عند استعمال سنيتينيب بالإضافة إلى إعطاء نتيجة إيجابية في معايرة dUTP الموسوم المُضاف للنهاية المكسورة بوساطة الانزيم الناقل الطرفي للنيكليوتيدات منقوصة الأكسجين (TUNEL assay) مما يعني أن حصول المـوت المبرمج هو أحد آليات عمله. أنتجت المركبات الفعالة الأخرى تليفاً وموتاً مبرمجاً للخلايا الطلائية والحشوية بدرجات متفاوتة. وأظهرت دراسات المجهر الإلكتروني حصول تليف وتغيرات تهتكيـة

في الخلايا الحشوية في العينات المعالجة بالمركبات الفعالة. وعند استخدام فحص حلقة الأبهر, منع مركب الكاريوفلين تكون أوعية دموية, وبالأضافة إلى عدم إحداث أي تأثير إحصـــائي ذي قيمـــه معتبره على الإباضة, والإنغراس, ووسط ونهاية الحمل وعلى الولادة الطبيعية في كل من مواقـــع الزرع, ومواقع الإدمصاص, وأعداد الأجسام الصفراء, وأعداد الأجنة والولادات ومستوى هرموني الإستروجين والبروجسترون في مصل الدم. لذا, قد تمثل المركبات الفعالـــة بدائل واعدة للعلاجات الطبية الحالية والتي تقلل من الخصوبة.